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THE NATURE OF ANTISTREPTOLYSIN S IN THE SERA OF MAN AND OF OTHER SPECIES. THE LIPOPROTEIN PROPERTIES OF ANTISTREPTOLYSIN S.

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EVIDENCE has been presented elswhere (Humphrey, 1949) to suggest that antistreptolysin S does not behave as a true antibody, and that inhibition of streptolysin S is a non-specific property of the serum of numerous species. Because of the interesting properties of streptolysin S, which in serum broth cultures appears to be a lipoprotein and in nucleic acid broth cultures to be a nucleoprotein (Herbert and Todd, 1944 ; Bernheimer and Rodbart, 1948), and because such non-specific inhibitors may play a part in natural resistance to disease, it seemed desirable to study further the nature of antistreptolysin S activity.

A possible clue appeared to lie in the observation of Hewitt and Todd (1939) that streptolysin S is powerfully inhibited by lecithin in the absence of protein, particularly since the levels of serum lipoids have been found to fall during acute disease and to rise to normal or supra-normal values during convalescence (Peters and Van Slyke, 1946), in a rather similar manner to the antistreptolysin S titres. An investigation of the effect of gentle extraction of the sera with lipoid solvents showed that such treatment did in fact remove much of the antistreptolysin S activity, but that the question was complicated by marked differences in behaviour between species and even between the sera of individuals within the same species. Further work on the behaviour of antistreptolysin S after proteolytic digestion of the serum, and of its distribution among serum fractions, has thrown some light upon its nature, although this is far from being completely revealed.

MATERIALS AND METHODS.

Streptolysin S was obtained from a strain of streptococcus (Group A, Type 11) which was known to produce streptolysin S, but not streptolysin O. Two preparations were used, as previously described, one being a horse serum extract of streptococci grown in serum broth, and the other a filtrate of a yeast nucleic acid broth culture. Sera were stored frozen at $-20°$ C. Antistreptolysin estimations were performed as described by Todd (1938) with slight modifications. In the case of streptolysin S the haemolysin was standardized against a human serum to which had been assigned an arbitrary value of 10 units per ml.

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Lipoid extractions.

Lipoid extractions were made with ether below − 25° C. as described by McFarlane (1948) or with alcohol-ether at − 10° C. as decribed by Hartley (1925). Three or four extractions were made with ether unless stated otherwise. All solvents were of Analytical Reagent grade and were free from peroxides and all but traces of aldehyde.

Lipoid phosphorus.

Lipoid phosphorus estimations were made by a modification of the method of Youngburg (Hawk, Oser and Summerson, 1947).

Cholesterol estimations.

Cholesterol estimations were performed by the digitonide precipitation method of Schoenheimer and Sperry as modified by Hawk, *et al.* (1947).

Total carbohydrates.

Total carbohydrate estimations were made by the orcinol method of Sørensen and Haugaard (1933) in terms of glucose.

EXPERIMENTAL.

Inhibition of streptolysin S by lecithin, cholesterol and cholesterol esters.

Preliminary experiments were performed to test quantitatively the inhibition of streptolysin S by lecithin and other lipoids. Antistreptolysin S estimations were made in the usual way, and it was found that, in the absence of serum, ovo-lecithin was powerfully inhibitory to both preparations of streptolysin S. A dispersion of highly purified lecithin at a concentration of 0·018 per cent in 0·9 per cent NaCl solution corresponded to 1 unit per ml. antistreptolysin S. When the streptolysin was diluted with normal rabbit serum, however, instead of buffer, the inhibitory effect of lecithin was reduced by more than 9/10ths, and it seems unlikely that lecithin, in the concentrations found in normal serum, could account for more than 0·5–1 unit antistreptolysin S per ml.

One sample of cholesterol, of which a very fine suspension was obtained, inhibited streptolysin S comparably with ovo-lecithin. This result was not in agreement with the findings of Hewitt and Todd (1939) and the experiments were repeated with recrystallized cholesterol. It was not found possible to obtain so fine a dispersion of the recrystallized cholesterol as of the first preparation, but with it no significant inhibition was obtained. Cholesterol esters (from wool fat) likewise caused negligible inhibition.

The effect of neutral fat was tested by measuring the antistreptolysin S of the serum of an individual taken while fasting and after a fat meal. Both the clear and lipaemic sera had the same antistreptolysin S titres.

The effects of ether and alcohol-ether extraction.

A number of sera, both from normal and sick people and from other animals, were extracted with ether below − 25° C. or with alcohol-ether at − 10° C. Treatment with ether removed nearly all free cholesterol, most of the ester cholesterol, and a variable fraction of the phospholipids. Alcohol-ether treatment removed nearly all detectable lipoids. The antistreptolysin S content of the sera were measured before and after extraction, and the results are given in

Table I. In order to ensure that the treatment was not so drastic as to destroy other known antibodies, these were also estimated in a number of cases by conventional methods. The results are summarized in Table II.

TABLE I.—*The Effect of Ether Extraction Upon the Antistreptolysin S Titres of Various Sera.*

(Ranges are given in parenthesis.)

Species.	Number of sera.	Mean antistreptolysin S units/ml. Before extraction.	After extraction.	Removed.
Human	10	8·9 (6–12·5)	3·6 (1·5–5·6)	60%
Horse	6	16·5 (10–27)	3·5 (2·4–6·4)	79%
*Horse (anti-*welchii*)	6	5·5 (4·8–7·2)	2·5 (2·2–2·8)	55%
Rabbit	2	5	1·3	74%
Rat	2	55	6	89%
Ox	1	23	2·9	87%
Sheep	1	12	3·1	74%
Plaice	1 pool	48	23	52%
Guinea-pig	3 pools	46	43	6%

* Sera preserved with 0·3 per cent tricresol.

Effect of Alcohol-ether Extraction.

Human	4	8·7 (6·5–11)	3·7 (3·1–5·4)	58%
Horse	2	18·5	4·3	77%
Guinea-pig	1 pool	36	17	53%

TABLE II.—*The Effect of Ether Extraction Upon the Titre of Various Antibodies in Sera.*

(Ranges are given in parenthesis.)

Species.	Number of sera.	Nature of antibody.	Mean value. Before.	After.	Removed.
Human	9	Antistreptolysin O units/ml.	303 (28–1200)	260 (21–1000)	14%
Horse	1	Ditto	100	100	0%
Horse*	1	Diphtheria antitoxin. Units/ml.	420	390	7%
Horse*	5	*Cl. welchii* β-antitoxin. Units/ml.	1300 (900–1800)	1250 (950–1800)	4%
Human	4	Iso-agglutinin titre	1 : 46	1 : 39	15%

The Effect of Alcohol Ether Extraction.

Human	5	Antistreptolysin O Units/ml.	872 (31–1200)	523 (20–810)	39%
Horse*	1	Diphtheria antitoxin. Units/ml.	420	340	19%

* Estimations by Dr. C. L. Oakley, Wellcome Physiological Research Laboratories, Beckenham.

From Tables I and II it is clear that whereas lipoid extraction has a relatively minor effect upon a variety of known antibodies, it causes a major reduction in the antistreptolysin S titres of all the sera examined (human, horse, rabbit, rat, ox) except for guinea-pig, and possibly plaice sera, in which considerable antistreptolysin S activity remained even after alcohol-ether treatment.

It was of interest to examine the ether extracts for antistreptolysin S activity. These were accordingly either recombined with the extracted sera or added to equivalent volumes of 0·9 per cent NaCl, and the ether was removed *in vacuo* over H_2SO_4. Both the saline and the serum preparations were very much more opalescent than the original sera, and a layer of fat formed on the surface after standing for some hours. Thus it was evident that the original lipoid complexes were not wholly restored.

Representative results are given in Table III in which the behaviours of antistreptolysin S and antistreptolysin O are contrasted. It will be observed that in each case the antistreptolysin S activity of the extracted lipoid only partly accounted for the difference between original and extracted sera, and that the reconstituted serum behaved with respect to antistreptolysin S as a simple mixture of the extracted serum and the lipoids. In the case of antistreptolysin O, however, the lipoid extracts had activities far greater than those of the original sera, yet the reconstituted sera were equivalent to the original sera. This rather puzzling observation is explained by the observation (Hewitt and Todd, 1939)) that *free* cholesterol is a very powerful inhibitor of streptolysin O. Thus lipoid extraction, while leaving the true antibody unchanged, frees cholesterol from the inactive protein complexes in which it occurs in serum. It is clear, however, that such complexes must be formed anew when the extracts are recombined, although this is not the case with the complexes concerned with antistreptolysin S activity.

TABLE III.—*Antistreptolysin S and O Contents of Sera Before and After Extraction with Ether, Compared with Titres of the Extracted Lipids Added to Saline and to the Ether Extracted Serum.*

		Original serum.	Ether extracted serum.	Extracted lipoids.	Reconstituted serum.
Human A	A.S.S.	10	4	1·5	5·8
	A.S.O.	28	28	450	53
Human B	A.S.S.	6·2	4	$<<1$	4·7
	A.S.O.	1200	1000	2000	1200
Horse (anti-scarlatina)	A.S.S.	13	2·5	<1	2·6
	A.S.O.	100	100	900	100

The effects of proteolytic digestion.

In order to disrupt the protein moiety of the lipoid complexes in serum use was made of proteolytic digestion. Of the available enzymes pepsin seemed suitable, since it would be inactive at pH 8·0, even in the presence of reducing agents, and since serum contains no natural inhibitor for it. Sera were treated with pepsin (B.D.H.) at pH 3 and at 37° C. until approximately 20 per cent of the protein nitrogen was no longer precipitable by tungstic acid. The sera were adjusted to pH 7, and the antistreptolysin S and antistreptolysin O contents were

measured before and after extraction with ether. Control samples were incubated under similar conditions without pepsin.

Through the kindness of Dr. C. L. Oakley there were available sera from horses immunized against *Cl. welchii* which contained considerable amounts of β-antitoxin, and which had been subjected to pepsin treatment so brief as to leave the antitoxin titres substantially unchanged (Harms, 1948), The antistreptolysin S and β-antitoxin of these sera were estimated before and after extraction with ether.

The results are summarized in Table IV. From this it will be seen that prolonged incubation with pepsin, actually tended to increase the antistreptolysin S values of the sera, although antistreptolysin O was largely destroyed. Furthermore, after such prolonged treatment almost all the antistreptolysin activity could be extracted by ether. From the control experiments without pepsin it appears that most of these effects were obtained by incubation at pH 3 alone.

TABLE IV.—*Effect of Pepsin Treatment upon Antistreptolysin S, Antistreptolysin O, and Anti-β-toxin Contents of Sera.*

(Mean values are given as percentages of those of the original sera.)

Treatment.	Type of serum	Number.	Antistreptolysin S. Before ether.	Antistreptolysin S. After ether.	Antistreptolysin O. Before ether.	Antistreptolysin O. After ether.
Prolonged with pepsin	Human	3	190%	9%	7%	Not done
	Horse	2	100%	6%	Not done	,,
Prolonged without pepsin	Human	2	100%	6%	1%	,,
	Horse	2	78%	6%	Not done	,,
					Anti-β-toxin	
Brief with pepsin	Horse	5	135%	62%	77%	71%

Brief incubation with pepsin also caused a rise in antistreptolysin S, but in this case there was no increase in extractability with ether. Presumably under such mild conditions (in which the β-antitoxin was largely unchanged) the lipoprotein complexes were only slightly disrupted.

The correlation between serum lipoids and antistreptolysin S titres.

The experiments described above suggested that lipoid-protein complexes were responsible for the greater part of the antistreptolysin S activity of sera, with the possible exceptions of guinea-pig and plaice sera. In order to test whether any direct correlation could be found between the presence of particular lipoid constituents and antistreptolysin S activity, the lipoid phosphorus and free and total cholesterol content of a number of sera from different species were estimated, both before and after extraction of lipoids. From Table V it is apparent that there was no correlation between antistreptolysin S titres and any of the serum constituents measured. It is particularly notable that the alcohol-ether extracted sera contained only very small amounts of phospholipid or cholesterol, and yet had considerable residual antistreptolysin S.

TABLE V.—*Correlation Between Antistreptolysin S and Serum Lipoid Content.*

Species.	No.	Antistreptolysin S. (units/ml.) Range.	Antistreptolysin S. (units/ml.) Mean.	Mean lipoid P. (mg./100 ml.)	Mean free cholesterol. (mg./100 ml.)	Mean total cholesterol. (mg./100 ml.)
(1) *Untreated sera.*						
Human	6	9–13	10	8·4	65	161
,,	8	7–9	7·8	6·3	37	138
,,	12	2–7	5·5	6·8	42	140
Horse	2	—	18·5	4·3	20	89
Rat	2 pools	—	55	1·4	0	31
Guinea-pig	1 pool	—	35	1·72	Not done	37
Sheep	1	—	12	3·2	,,	53
Plaice	1 pool	—	50	9·5	,,	109
Dog fish	,,	—	5	3·1	,,	104
Spider crab (whole blood)	,,	—	1·2	0·24	,,	<10
(2) *Ether extracted sera.*						
Human	4	3·9–5·6	4·5	7·2	2	18
Horse	2	—	6	2·5	11	21
Rat	2 pools	—	6	1·3	0	5
Sheep	1	—	3·1	1·9	Not done	<10
(3) *Alcohol-ether extracted sera.*						
Human	3	—	3·7	0·5	0	7
Horse	2	—	4·4	0·25	0	0
Guinea-pig	1 pool	—	1·7	0	0	0

The antistreptolysin S content of serum fractions.

Effect of dialysis.—The distribution of antistreptolysin S activity in serum between the euglobulin fraction (precipitated by prolonged dialysis in the cold against distilled water saturated with CO_2) and the remainder was studied in several sera (Table VI). Somewhat surprisingly it was found that the two

TABLE VI.—*Distribution of Antistreptolysin S Between Euglobulin Fraction and Residue in Various Sera.*

Serum.	Original titre (units/ml.).	Per cent original activity in Euglobulin.	Per cent original activity in Remainder.	Recovery.
Human	12	7	81	98
Ox	23	130	108	238
Guinea-pig	32	146	148	264
Horse 7629	27	80	30	110
,, ,, (after ether extraction)	4	58	19	77
Horse 7637	10	45	100	145
,, ,, (after ether extraction)	3·3	70	30	100

fractions of ox, guinea-pig and horse sera taken together had more antistreptolysin S activity than the original serum. Furthermore, the two horse sera differed sharply from one another—that with a high antistreptolysin S having relatively a much larger activity in the euglobulin than the other, while the activity of the remaining fractions was about equal. After ether extraction the two horse

sera behaved similarly, and their euglobulin fractions contained almost 3/4 of the residual antistreptolysin S.

These experiments demonstrated that the separate antistreptolysin S activities of serum components were not necessarily equal to the activity of the original serum, and that not only were there differences between species but also between individuals within a species. It was nevertheless decided to attempt to follow the behavious of antistreptolysin S in human serum fractionated by ammonium sulphate and by electrophoresis.

Ammonium sulphate fractionation.—Two normal human sera, before and after extraction with ether, were fractionated with ammonium sulphate solution (buffered at pH 6·7) in the cold. The fractions were freed from ammonium sulphate, by dialysis in the cold first against distilled water and then against 0·9 per cent NaCl. The antistreptolysin S and O, and the nitrogen, total cholesterol, lipoid phosphorus, and total carbohydrate content of each fraction were then estimated. Table VII shows the distribution of antistreptolysin S and antistreptolysin O between the fractions. The percentages are given in terms of the original serum activity, and the differences between the two sera are largely due to poorer recovery of activity in serum B than serum A.

TABLE VII.—*Percentages of Original Antistreptolysin S and O Activity Recovered in Different Fractions of Human Serum.*

	Per cent saturation with ammonium sulphate.							
	0–33		33–40.		40–50.		50–80	
Serum.	S.	O.	S.	O.	S.	O.	S.	O.
Human A	7	77	9	14	16	9	59	0
Human A (ether extd.)	7	64	7	9	7	2	7	0
Human B	5	53	6	31	8	16	58	0
Human B (ether extd.)	3	30	4	15	4	8	4	0

Electrophoretic Fractions.

	γ-glob.		β- + γ-glob.		α-glob. + alb.		Albumen.	
Antistreptolysin	S.	O.	S.	O.	S.	O.	S.	O.
Human C	(?5)	58	(?12)	42	16	0	67	0

It is clear, however, that (in contrast to antistreptolysin O) the antistreptolysin S activity is mainly in the albumen containing fractions, and that it is from these that the activity is removed by treatment with ether.

No quantitative correlation was found (Table VIII) between the antistreptolysin S and the total cholesterol, the lipoid phosphorus, the protein nitrogen or the total carbohydrate in the fractions of the unextracted sera, and although after ether extraction there was apparently a fair correlation between carbohydrate content and antistreptolysin S in serum A, there was more variation in serum B.

In contrast to the results obtained with the human sera, similar fractionation of pooled guinea-pig serum, before and after extraction with alcohol-ether, showed that the antistreptolysin S activity was initially high both in the albumen and globulin fractions, and that after extraction the activity of the globulin fraction was diminished more than that of the albumen.

TABLE VIII.—*Correlation of Antistreptolysin S and Serum Constituents in Fractions of Serum A.*

	Antistreptolysin S in units per mg. of			
Before extraction.	Nitrogen.	Cholesterol.	Lipoid P	Total carbohydrate
0–33% Am. Sulph.	0·33	8·1	136	5·4
33–40% ,, ,,	0·78	6·8	238	8·9
40–50% ,, ,,	1·59	9·1	200	16·5
50–80% ,, ,,	1·06	5·2	107	22·7
After ether extraction.				
0–33% Am. Sulph.	0·29	17	84	5·0
33–40% ,, ,,	0·37	9	70	6·5
40–50% ,, ,,	0·79	38	64	7·0
50–80% ,, ,,	0·14	10	37	6·2

Electrophoretic fractionation.—Through the kindness of Dr. C. L. Oakley an electrophoretic separation was performed at the Wellcome Research Laboratories on a sample of normal human serum. The separation was carried out at pH 8·0 in phosphate buffer, $\mu = 0{\cdot}2$. Albumen, albumen + α-globulin, β–+γ-globulin and γ-globulin fractions were examined for antistreptolysin S and O.

The results were in agreement with those found with the ammonium sulphate fractions, except in one respect. It was found impossible to measure the antistreptolysin S activity of the fractions containing γ-globulin when yeast nucleic acid broth streptolysin was used, irregular results with apparently very high titres being obtained despite several repetitions of the assay. When serum streptolysin, which in other estimations behaves in the same way as nucleic broth streptolysin, was used for the assay end-points were obtained, but the apparent antistreptolysin S activities both of the γ-globulin and of the β–+γ–globulin fractions were still greater than the total activity of the original serum. Similar phenomenona, though less well marked, were observed when estimating antistreptolysin S on separated ox and guinea-pig euglobulin fractions, and it appears that the combination between γ-globulin and streptolysin S is very much firmer in the absence of other serum components.

The effect of acid, alkali and heat.

It has already been mentioned (Table IV) that incubation of serum at pH 3 for 48 hr. caused only a small decrease, if any, in the antistreptolysin S content. Other samples of normal human serum—untreated, ether extracted and reconstituted after ether extraction—were kept at pH 9–10 for 3 days in the cold without significant change in antistreptolysin S. The same samples were heated at 65° C. for 1 hour, also without effect upon the antistreptolysin S.

Heating of normal and ether extracted horse serum at 100° C. for 5 minutes caused coagulation. The clear fluid which separated from the coagulum in both cases had considerable antistreptolysin S activity, approximately equal to that of the unheated ether-extracted serum. This activity was shown to be associated with uncoagulated protein, being reversibly precipitated by trichloracetic acid, and was not affected by further extraction with ether.

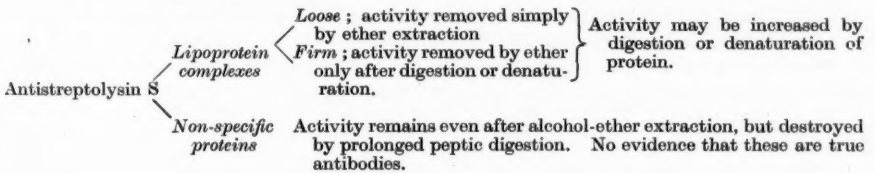
The effect of varying treatment with ether.

It had been observed that successive extractions of serum with ether below − 25° C., up to four in number, all removed antistreptolysin S activity, though in diminishing amounts. It was of interest to note the effect of saturation of serum with ether, with gentle shaking, followed by removal of ether by evaporation on a pump so as to leave behind all the original serum components. When a horse and a human serum were thus treated at room temperature their antistreptolysin activities fell to 55 per cent and 71 per cent, and when treated below − 25° C. to 26 per cent and 40 per cent respectively. Thus it is evident that the lipoprotein complexes concerned in antistreptolysin S activity are extremely labile to ether and are irreversibly broken simply by saturating at room temperature.

DISCUSSION.

The evidence presented above supports the suggestion made on serological grounds that antistreptolysin S is not an antibody in the accepted sense, and furthermore that its nature varies between species, and even between individuals. In human serum most of the antistreptolysin S activity is carried by a labile lipoprotein complex associated with the albumen fraction, but the globulin fractions also have some activity, most of which is retained after gentle treatment with fat solvents.

Mild denaturation or peptic digestion may increase the antistreptolysin S activity by loosening the lipoprotein complexes without destroying that part of the proteins which carries the residual antistreptolysin S activity after ether extraction. More extensive digestion of the proteins, however, destroys this residual antistreptolysin S. Schematically this may be represented as follows :


Antistreptolysin S
Lipoprotein complexes
Loose ; activity removed simply by ether extraction
Firm ; activity removed by ether only after digestion or denaturation.
Activity may be increased by digestion or denaturation of protein.
Non-specific proteins
Activity remains even after alcohol-ether extraction, but destroyed by prolonged peptic digestion. No evidence that these are true antibodies.

The same scheme would also cover horse sera, with the proviso that in some sera a considerable amount of active lipoprotein complex may be found in the euglobulin fraction. Insufficient evidence was obtained for generalization about other species, but in guinea-pig serum, which behaved differently from other sera, lipoprotein complexes are either unimportant in this connection or else are so stable that they are unaffected by ether treatment.

Concerning the detailed nature of the inhibitors mainly negative evidence has emerged, for there was no apparent correlation between antistreptolysin S and any of the serum constituents examined. The methods of fractionation used were too crude to permit identification of the protein, or of the lipoprotein factors, which were included in the globulin fractions. Some information was gained by examination of the streptolysin S inhibition by human serum fractions prepared for another purpose by the cold ethanol fractionation method of Cohn and his colleagues (Edsall, 1947). Considerable inhibition was caused by the fractions containing the β_1 lipoprotein (Fraction III_0), the α_1 lipoprotein (Fraction IV_1),

and by the γ-globulin containing Fraction (II + III_w). The albumen fraction, however, presumably because of the alcohol treatment involved in fractionation, caused negligible inhibition. Fraction II and III (which contains the inhibitory III_0 and II + III_w) was notably less active than either of its constituent fractions demonstrating once again that inhibition of streptolysin S is a complex phenomenon, and that the affinity of a particular serum component may depend upon the presence or absence of others. Greater knowledge of streptolysin S itself is required before the exact mechanism of its inhibition can usefully be considered.

It may be of interest to compare antistreptolysin S with some other inhibitors of enzymes or toxins known to occur in normal sera. Duthie and Lorenz (1949) have recently studied trypsin inhibitor, and an inhibitor of the proteases of certain non-pathogenic bacteria, and have reviewed earlier work on these substances. Trypsin inhibitor is found mainly in the albumen fraction, although some activity was present in all fractions of serum examined. Unlike antistreptolysin S it is relatively unaffected by ether treatment, although much diminished by chloroform. The bacterial protease inhibitor was found to be a labile component of the globulin fraction, being present in varying amounts in different species. Plasmin inhibitor, studied by MacFarlane and Pilling (1946) and others, appears to resemble trypsin inhibitor in its behaviour. Among inhibitors of haemolysins may be mentioned an inhibitor of θ-toxin of *Cl. welchii* (private communication from Dr. C. L. Oakley), and a non-specific inhibitor of streptolysin O found by Packalén (1948) in sera from cases of hepatitis. The latter appears to be a lipoprotein present in α- and β-globulin fractions.

Normal sera also contain an inhibitor of hyaluronidase (McClean, 1942) and an inhibitor of the haemagglutinating and certain other properties of influenza virus which is found mainly in the β_1 globulins (McCrea, 1946 ; Hirst, 1949). In connection with this so-called " Francis inhibitor " it has been suggested by Smith and Westwood (1949) that such inhibitors may be biologically important in determining natural resistance to particular infectious agents. It is possible that the widespread occurrence of antistreptolysin S may account for the apparent unimportance of streptolysin S production in determining the virulence of streptococci.

SUMMARY.

(1) Treatment of sera with ether or alcohol-ether, in such a manner as to cause only minor changes in the titres of known antibodies, causes a major reduction in antistreptolysin S titre. This phenomenon was observed in the sera of man, horse, rabbit, rat, ox, sheep, but was less marked in guinea-pig and plaice sera.

(2) From a study of the effects on antistreptolysin S of peptic digestion of sera and of their fractionation by various methods, it is concluded that in man and the horse antistreptolysin S activity is a complex phenomenon, mainly dependent upon labile lipoprotein components associated with the albumen fraction.

(3) The antistreptolysin S activity of separated serum components may be greater than that of the original serum, presumably because some of the groups capable of linkage with streptolysin S are normally linked with one another.

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EXPERIMENTAL BERYLLIUM POISONING.

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THE work to be described in this paper was begun in January, 1947, to provide information on the toxicity of beryllium and its compounds. Hyslop, Palmes, Alford, Monaco and Fairhall (1943), in a long report, stated that no particular toxicity had been established for beryllium, and that whatever toxicity had been found with the salts of beryllium was due to the toxicity of the acid radicle and the hydrolysis of the salts. These writers had made an extensive review of the literature, but failed to emphasize the importance of earlier work by Siem (1886) and Comar (1935) which demonstrated the toxic properties of beryllium. When our preliminary experiments demonstrated beyond doubt that beryllium was a very toxic element, it became possible to assess the results of previous workers, and to explain many of the discrepancies and contradictions found in the literature. Hyslop *et al.* (1943) include in their report a very complete bibliography, and no useful purpose will be served by providing a similar list in this paper. It should,

however, be emphasized that confusion has arisen because both soluble and insoluble compounds have been used, and because no serious attention has been given to the varying effects produced by different routes of administration.

When given intravenously the soluble salts of beryllium have always shown themselves to be toxic in doses so small that neither the acidity of the solution nor the properties of the anion could be held responsible for the liver necrosis and death of the animal. Our experiments have confirmed the earlier ones of Siem (1886) and Comar (1935), and in a recent paper Scott (1948) has described the pathological lesions produced by beryllium administered intravenously.

Having established that beryllium was in fact toxic, we carried out further work in an attempt to find out more about the mode of action of beryllium, and the genesis of the very profound tissue reactions that it produced.

METHODS AND MATERIALS.

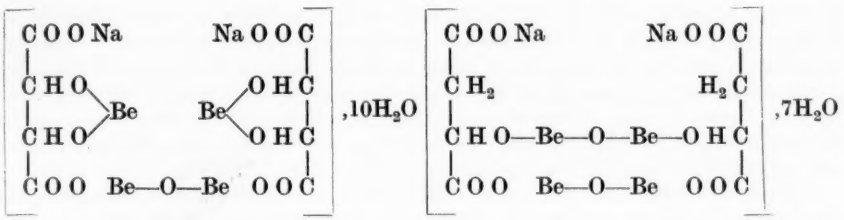
A method suitable for the microdetermination of beryllium in biological materials has been described elsewhere (Aldridge and Liddell, 1948). It is a long and difficult method, requiring considerable care and experience before reproducible results can be obtained. For these reasons the number of determinations made had to be limited. Further, the method cannot be used for determining beryllium in bone or faeces; low values obtained in control experiments with these materials were assumed to be due to the mechanical entrainment of beryllium compounds in the large amount of calcium sulphate deposited in the sulphuric acid digest.

For some of the work a roughly quantitative spectrographic method was employed, and a few spectrographic determinations of beryllium in bone were also carried out for us at the Atomic Energy Research Establishment, Harwell.

Standard histological methods were used for the examination of the tissues. Much valuable information was obtained from the use of a special histochemical method for staining beryllium in fixed tissues. The method will be described in full elsewhere.

Rats—albino and hooded—were bred at the Animal Farm, Chemical Defence Experimental Establishment. The greater bulk of the work was done with these animals, which were in good condition and free from intercurrent disease. Mice and rabbits were obtained mainly from dealers. The rabbits varied very considerably, and many were heavily infected with coccidia.

Beryllium compounds used were prepared from metallic beryllium of at least 99·9 per cent purity. Beryllium sulphate, oxyfluoride and lactate were used and, in addition, ammonium beryllium oxalate and sodium beryllium tartrate and malate were prepared for certain experiments. Beryllium lactate is a basic lactate whose composition varies with the conditions of preparation. For this reason all samples were analysed for their beryllium content. It is freely soluble in water, giving a clear solution of about pH 5·5. Ammonium beryllium oxalate is a double salt in which beryllium is ionized. Beryllium also forms complex compounds with sodium tartrate and malate. In these salts beryllium is contained within the anion and is not ionized. The compounds are freely soluble, but the beryllium is not precipitated by phosphate. They have been assigned the following formulae (Parsons, 1909):



Every sample of beryllium lactate, tartrate and malate was analysed for beryllium.

All the earlier work was carried out with beryllium lactate because solutions of this salt are only slightly acid and the anion can be considered completely non-toxic.

RESULTS.

Toxicity of Soluble Beryllium Compounds by Intravenous Injection.

Solutions of beryllium lactate and sulphate were injected intravenously into rabbits, rats and mice. When the dose of beryllium is equivalent to 0·5–1 mg. per kg. body weight, death of the animal usually follows 1 to 4 days later. An average lethal dose of the same order was reported by Siem (1886) and Comar (1935) with cats, dogs and rabbits injected with beryllium tartrate, lactate and sulphate.

The LD50 for beryllium lactate was calculated for mice (20 g.) and rats (200 g.). The figures are presented in Table I. Young rats are somewhat more resistant than older animals.

TABLE I.—*The Mortality Rates and LD50 for Beryllium Lactate Injected Intravenously into Rats and Mice.*

	Mice. Weight 20 g.		Rats. Weight 200 g.	
	Dose mg. Be/kg.	Deaths/total injected.	Dose mg. Be/kg.	Deaths/total injected.
	0·25	7/20	0·3	2/20
	0·5	13/20	0·6	25/40
	0·75	19/20	0·9	18/20
LD50 :	0·34 mg./kg.		0·53 mg./kg.	
Fiducial limits :	0·23—0·42		0·44–0·60	

Rabbits seemed slightly more sensitive in that a dose of 0·5 mg. of beryllium per kg. killed all of the 37 animals injected, but the high incidence of coccidial infection with attendant liver cirrhosis may have been partly responsible.

There was no immediate reaction to the injection of an average lethal dose, but within 18 hours the animals were obviously ill, sitting quietly and refusing food. This condition persisted until death. In the case of rats and mice death took place unobtrusively, but in many rabbits a short series of violent convulsions immediately preceded death. The rats, if they survived 3 days or longer, became jaundiced.

At post mortem all animals showed a striking degree of liver damage, obvious

to naked eye inspection. If death was early the liver was red, friable and haemorrhagic, but in later deaths the liver became yellow and soft. In rabbits there was a variable amount of haemorrhagic peritoneal exudate. The renal surfaces often had superficial haemorrhages, as did also the pleural surfaces of the lungs. These changes were only seen in the rabbits, and may have been partly the result of the terminal convulsions. One other noticeable feature was the delayed clotting time of the blood from animals dying after beryllium poisoning.

There was very little variation in the general picture of beryllium poisoning seen among animals which were the subject of experiments spread over more than 2 years.

The claim of Hyslop *et al.* (1943) that the toxicity of beryllium compounds was connected with the properties of the anion or of the salt as a whole, rather than being a specific property of beryllium, seemed to be adequately discountenanced by these experiments with beryllium lactate. The toxicity of a number of beryllium salts was compared in an experiment with mice in which the animals each received 10 μg. of beryllium in solutions of different salts. The results, presented in Table II, show that there is no marked difference in the toxicity of the different salts with the exception of sodium beryllium malate. It is possible that the explanation may be in the rate of breakdown of this salt to give a slow release of beryllium into the tissues.

TABLE II.—*The Comparative Toxicity of Different Salts of Beryllium.*

Animals : Mice (20 g.).
Number per group : 30.
Dose of beryllium injected : 10 μg./mouse.
Volume : 0·1 ml.
Route : Intravenous.

Salt.	Mortality per cent.
Beryllium sulphate	77
Beryllium oxyfluoride	88
Beryllium lactate	66
NH_4 beryllium oxalate	67
Na beryllium tartrate	80
Na beryllium malate	13

The toxicity of beryllium was also compared to that of lithium, magnesium, calcium and aluminium injected intravenously into mice as solutions of their lactate. Whereas a dose of 20 μg. beryllium killed every mouse, the molar equivalent of 40 μg. beryllium as lithium, calcium or aluminium did not kill a single mouse. Magnesium at this level produced acute symptoms which were occasionally fatal within a few minutes, but the surviving animals recovered completely.

The Fate of Beryllium after the Intravenous Injection of its Soluble Salts.

The method of estimation devised by Aldridge and Liddell (1948) made it possible to determine the amount of beryllium down to 0·5 μg. in all tissues except bones and faeces. A number of determinations were therefore made of the amount of beryllium held in the different tissues at varying times after the intravenous injection of beryllium salts.

In a preliminary experiment a rabbit was injected with 7 mg. of beryllium per kg. as a solution of beryllium lactate. Spectrographic analysis of the blood showed that within 6 hours all but a trace of beryllium had left the circulation. The organs of this animal were analysed by the chemical method after death, 44 hours after the injection. The blood, heart, lungs, liver, spleen, kidneys, skeletal muscle and brain were examined. About 30 per cent of the total beryllium injected was accounted for, and of this, 40 per cent was in the liver and 48 per cent in the spleen.

A further series of experiments was carried out with mice. In each case the animal received 80 μg. beryllium in 0·1 ml. of a solution injected into the tail vein. Animals were killed at different times after injection and the organs analysed for beryllium. The results of several experiments are presented in Table III. After a dose of this size mice do not live as long as 48 hours, and so it was not possible to see whether beryllium concentrated in the spleen after 24 hours.

TABLE III.—*The Beryllium Content of Organs of Mice Killed at Different Times After the Intravenous Injection of* 80 μg. *of Beryllium.*

	Time after injection of beryllium.			
Organ.	5 minutes.	1 hour.	2 hours.	24 hours.
Liver	10·8 (3)	24·8 (8)	29·5 (6)	22·0 (7)
Spleen	0 (3)	2·8 (8)	2·0 (6)	2·1 (7)
Kidney	2·1 (3)	0·8 (8)	0·6 (6)	0·7 (7)
Lung	0 (3)	1·2 (7)	0·6 (6)	0·6 (6)
Gut	0 (3)	3·3 (8)	3·3 (6)	2·3 (7)
Percentage beryllium recovered :				
a. Total	16	40	45	34
b. In liver	13·5	30	31	27·5

Beryllium (μg.)expressed as average of a number of experiments (in brackets).

In addition the total beryllium recovered is expressed as a percentage of the amount injected, and this never exceeds 50 per cent. The heart, brain, skin and skeletal muscles were examined, but never contained any measurable amounts of beryllium. The tail was also analysed to check any possible loss of beryllium round the vein at the time of the injection but only an occasional trace was recovered. In a later experiment 12 mice were each injected with 80 μg. of beryllium into the tail vein. Four were killed 1/2, 6 and 24 hours after injection. The total beryllium in the skeleton of each mouse was determined spectrographically, and amounts varying from 2·1 to 8·5 μg. per mouse were found. There was no evidence of any accumulation of beryllium in the skeleton within the first 24 hours of a single intravenous injection. The total beryllium in the skeleton never accounted for more than 10 per cent of the injected beryllium.

The excretion of beryllium after the intravenous injection into rats was determined and did not exceed 10 per cent of the total injected, even in animals that lived for 72 hours.

Although we have not found it possible to trace all the injected beryllium, it is noteworthy that of the amount that can be recovered, by far the greater part is in the liver. The figures for mice are given in Table III. The figures for rats

are similar (Table IV). However, in early experiments with rats, in which a small weighed sample of the liver was taken, the results were extremely inconsistent. It was then discovered that the concentration of beryllium in different parts of the liver varied very considerably, and so the content of the whole liver could not be calculated from a determination made on a small sample. In Table IV the figures are given for the concentration of beryllium in the four main lobes of the livers of 7 rats. The variations in concentration in a given animal are considerable, but there is no fixed ratio of the amounts of beryllium in the different lobes. This suggests that there is no anatomical basis to explain these variations. The total amount of beryllium found in the liver 20 hours or more after injection was about 30 per cent of the total injected.

TABLE IV.—*The Concentrations of Beryllium in the Separate Lobes of the Livers of Rats Injected with* 200 μg. *Beryllium Intravenously and Killed at Intervals After.*

Animal No.	1	2	3	4	5	6	7
Time after injection	7 min.	15 min.	4 hr.	20 hr.	20 hr.	20 hr.	48 hr.
Concentration of beryllium (μg./g. liver).							
Lobe: Right anterior	1·7	6·6	10·0	8·2	8·4	10·3	6·2
Left anterior	5·9	4·9	9·9	5·2	7·7	5·1	3·6
Right posterior	3·9	5·0	15·5	16·5	4·3	3·3	3·3
Left posterior	6·0	7·5	11·5	7·4	9·1	4·5	4·6
Total beryllium in liver (μg.)	29	33	41	53	52	69	47
Percentage of injected beryllium	14·5	16·5	20·5	26·5	26	34·5	23·5

(Beryllium as μg./g. of liver.)

The distribution of beryllium in the liver of the rabbit was found to be more nearly equal in all parts. The analysis of 5 samples taken from different lobes of a liver of a rabbit, dying 48 hours after the injection of 0·2 mg./kg., showed a variation of only 1·55 to 1·93 μg./g. in the concentration of beryllium. Again, about 40 per cent of the total beryllium injected could be recovered from the livers of rabbits 24 hours or more after the injection.

The results of the tissue analyses for beryllium indicated that while some 40 per cent of injected beryllium could not be traced, the greater part of the remainder collected in the liver and remained there until the animal died. Only a small percentage was excreted, and there was no evidence of any concentration in bone over the short period of these acute experiments.

The Tissue Reactions to Beryllium.

The tissue reactions to the intravenous injection of beryllium salts have been studied by a combination of the usual histological staining methods with a special histochemical method devised by Denz (1949). With the aid of the latter method it was possible to stain the tissues specifically for beryllium. The method consists of forming a coloured compound with beryllium and the dyestuff " naphthocrome Green B " under conditions in which iron, aluminium and other elements normally present in the tissues do not react with the stain. The validity of the results can be shown by a comparison of the intensity of the staining seen in different organs with their beryllium content estimated chemically, e.g. :

	Liver.	Spleen.	Kidney.	Lung.	Heart.
Concentration of beryllium (μg./g. wet tissue)	29·5	2·0	0·6	0·6	0
Intensity of beryllium stain	+++	++	+	−	−

The histochemical method is not sufficiently sensitive to detect beryllium in the tissues after the injection of a single lethal dose, and as a rule histochemical tracing was carried out after the injection of 8 M.L.D.

The distribution of beryllium after 8 M.L.D. might be very different from the distribution after the injection of 1 M.L.D. However, it is just possible to detect beryllium in the tissues after 2 M.L.D., and the distribution is the same as after 8 M.L.D.

The distribution of beryllium and the histological changes in the different tissues are described in detail below :

Liver.

Within a few minutes of the intravenous injection of beryllium lactate beryllium can be seen within the arteries and veins of the liver, and the parenchyma takes on a pale green tint. Within 10 minutes beryllium begins to collect in the sinusoids. By 30 minutes the diffuse staining has reached a maximum, and thereafter decreases slowly. But the staining of beryllium in the sinusoids increases in intensity up to 2 hours, and persists for 24–48 hours while pathological lesions are developing. A few hours after the injection of beryllium the liver presents a characteristic picture, with the pale diffuse green staining of the parenchyma speckled with the darker green of focal concentrations within the sinusoids. These focal concentrations of beryllium appear to consist of protein masses staining uniformly for beryllium and lying within the lumen of the sinusoids. They are found predominantly in the midzone of the lobule, though occasional aggregations are seen in the central and peripheral zones. There is some evidence of the phagocytosis of these beryllium protein masses by the Kupffer cells, but this does not account for the focal accumulations of beryllium.

Pathological changes appear in the liver within three hours of the intravenous injection of a dose of 1 mg. beryllium per kg. The normal pattern of the liver is replaced by a uniform field of swollen cells which have become hexagonal in outline as a result of common pressure. This generalized reaction disappears within 24 hours. Instead, the sinusoids of the midzonal regions now become dilated and many are grossly distended. Focal lesions in the liver cells become conspicuous. These focal lesions may be detected within 6 hours, and consist of small groups of necrotic parenchymatous cells. The cytoplasm of these cells becomes pale and vacuolated, the cell outlines are indistinct and the nuclei either hydropic or small, dark and pyknotic. Groups of damaged cells sometimes form rosettes around midzonal sinusoids.

These changes are taking place while the focal concentration of the beryllium in the sinusoids is increasing, and around these areas focal necrosis develops. Within 24 hours the general reaction of the liver has subsided, and some time before this the general staining of the parenchyma with beryllium has faded. Meanwhile, around the tiny focal aggregates of beryllium in the sinusoids cellular necrosis has started. By the end of 24 hours these focal aggregates are beginning to fade as the beryllium begins to spread in an ever-widening circle into the

damaged tissue round about. The liver cells begin to disintegrate, and the nuclei either break up into pyknotic fragments or, more commonly, just fade away.

The connective tissue of the portal tracts becomes oedematous and infiltrated with round cells. The sinusoids are invaded by large numbers of polymorphonuclear leucocytes. The Kupffer cells show signs of phagocytic activity but no sign of active proliferation.

By 48 hours the focal lesions involving the midzone of the lobules are fully developed. In some places eosinophilic masses infiltrated by neutrophils are still present; in others the necrotic material has entered the central veins, leaving behind blood-filled spaces. The reticulum persists until softening is complete and the blood spaces have appeared.

In animals that live for 72 hours or more the extent of the liver destruction is very considerable, and a constant feature of the necrosis produced by beryllium is the persistence of a collar of liver cells, as a single layer, round the central veins and portal tracts.

Kidney.

Within 15 minutes of injection beryllium can be seen in the arcuate vessels and *vasae recti*. It disappears from the former within 15 minutes, but can be seen in the inter-lobular veins up to 30 minutes. Only in isolated cases has it been seen within single glomeruli, and it has not been found in the capillary network surrounding the tubules. Beryllium is, however, found in the medulla of all kidneys examined 30 minutes after injection, lying within the *vasae recti*. These vessels, which are arranged in bundles, contain hyaline eosinophilic masses of plasma protein that stain strongly for beryllium. This staining may be seen up to 4 hours after injection and then disappears gradually, so that only in exceptional cases can any beryllium be seen in the *vasae recti* after 24 hours. Unlike the liver, histological changes cannot be detected in the kidney during the first 24 hours, and by the time they do appear all but traces of the injected beryllium have left the kidney. During the second 24-hour period changes take place in some of the proximal convoluted tubules. The tubular epithelium loses its affinity for stains and becomes ragged in outline, while the lumen contains cytoplasmic and nuclear debris, but complete protein casts are not formed at this stage. These lesions are best seen in tissues fixed in Helly's fluid and stained by Heidenhain's azocarmine-aniline blue method. The damaged proximal convoluted tubules with their pale, flattened epithelium and wide lumen superficially resemble distal convoluted tubules, but the damaged tubules can be recognized by their ragged cytoplasm and their position around glomeruli. At 48 hours the changes in the proximal convoluted tubules are well developed. The epithelium of the damaged tubules is no longer columnar, but has become so flattened that the surviving nuclei bulge into the lumen. The protein casts in the lumen of damaged tubules stain brilliantly with azocarmine, and, surrounded by a thin white collar of damaged epithelium, contrast strongly with the purple of undamaged tubules. The damaged tubules are found scattered among normal tubules, and a focal lesion which may give the impression of damage to many different tubules is, in fact, made up of lesions to the same tubule.

At 72 hours the damaged tubules are easier to see owing to the formation of more casts, but the number is not increased.

The extent and exact site of the damage in individual nephrons has been

investigated by examining serial sections and reconstructing nephrons in plasticine. No changes have been detected in the glomeruli. The tubular damage starts at the point of origin from the glomerular capsule. Damage to the neck of the tubule is always slight, while the most severe damage is in the convoluted part of the proximal convoluted tubule. As the straight part is reached the severity of the lesion decreases, and by the time the loop of Henle is reached the epithelium once again appears normal. The rest of the nephron is free from pathological change. The severe lesions affect only a small number of nephrons, but if apparently normal nephrons are carefully followed in serial section, lesions will commonly be found in the neck and in the convoluted part of the proximal convoluted tubules.

Neither the chemical nor histochemical method is able to detect any appreciable quantities of beryllium after the first few hours of an intravenous injection. It is possible that the attempted excretion of small quantities of beryllium throughout the whole period of survival may account for the damage, but the localized nature of the lesions suggests that the main injury occurs over a relatively short period of time.

Spleen.

Diffuse beryllium staining of the red pulp of the spleen appears within 5 minutes of the injection and in 15 minutes is well developed. This diffuse staining reaches a maximum within 30 minutes, and then declines slowly, but is still present after 48 hours. Concentration of beryllium takes place in the marginal zone between the red and white pulp. This is clearly marked after 15 minutes and persists. The Malpighian bodies are entirely unstained and clearly outlined by the deeply stained marginal zone. This concentration of beryllium is due to the accumulation of the beryllium protein masses within the sinusoids of the marginal zone. The same sinusoids are clearly outlined by an intravenous injection of Indian ink into the living animal. As the diffuse staining of the red pulp fades, foci of more intense staining develop within the red pulp. Histological changes are first seen in the marginal zone between the red and white pulp. Small focal lesions develop round the sinusoids. Groups of cells become ill-defined and autolysed. Within 24 hours these small lesions have spread to involve the whole of the marginal zone, which is now shrunken and empty of large lymphocytes. Similar small lesions develop round the sinusoids in the red pulp with destruction of the lymphocytes and other nucleated cells. Infiltration of the damaged spleen by granulocytes begins within a few hours of the injection of beryllium.

At 72 hours the appearance of the spleen is characteristic of acute beryllium poisoning. The cellular white pulp stands out in strong contrast with the structureless red pulp, which is packed with red cells and free of lymphocytes.

The surviving nucleated cells in the red pulp are endothelial and reticulum cells, some nests of primitive red and white blood cells, and a relatively large number of mature granulocytes that have infiltrated the damaged tissue. The underlying reticular structure remains unaltered.

Changes in the white pulp are less extensive. It becomes relatively reduced in volume, and the lymphocytes are loosely arranged instead of being packed tightly together.

The histological evidence is that beryllium tends to collect in the sinusoids of the spleen, especially in the marginal zones, and round these aggregates of beryl-

lium cellular destruction starts and then spreads rapidly throughout the red pulp. Beryllium never enters the white pulp, and changes here are limited to a reduction in the number of lymphocytes.

Other organs.

No changes attributable to beryllium poisoning have been found in the brain, heart, voluntary muscle, lung, intestine, pituitary, thyroid, parathyroid, pancreas, ovary, testis, thymus and lymph nodes. Histochemical staining failed to show any evidence of the accumulation of beryllium in any of these organs, although, of course, it can be seen within the larger vessels of all organs up to 15 minutes after an injection. Thirty minutes after the injection of 8 M.L.D. no beryllium is visible in the lumen of blood vessels except in the kidney.

Changes have been seen in the adrenals with shrinkage of the zona glomerulosa and other less definite changes. These changes are probably associated with the physiological response to damaged tissues elsewhere.

Peripheral blood.

The injection of beryllium salts in lethal or sublethal doses produces a sharp rise in the circulating granulocytes and a fall in circulating lymphocytes. After sublethal doses the granulocytes return to normal from the fourth day. The figures from rats injected with lethal doses of beryllium are presented in Table V.

TABLE V.—*The Effect of a Lethal Dose of Beryllium on the Numbers of Circulating Granulocytes and Lymphocytes.*

(1000 per c.mm.)

	Granulocytes.	Lymphocytes.	Number of animals.
Controls	$3 \cdot 49 \pm 1 \cdot 64$	$15 \cdot 31 \pm 5 \cdot 47$	77
Hours after beryllium:			
1 to 8	$10 \cdot 28 \pm 0 \cdot 67$	$11 \cdot 47 \pm 4 \cdot 86$	16
24	$14 \cdot 65 \pm 5 \cdot 40$	$10 \cdot 07 \pm 4 \cdot 36$	21
48	$22 \cdot 87 \pm 4 \cdot 71$	$7 \cdot 82 \pm 3 \cdot 30$	13
72	$7 \cdot 20 \pm 7 \cdot 25$	$4 \cdot 45 \pm 3 \cdot 26$	20

The red blood cells are not affected, nor is there any evidence from a study of the bone marrow of a disturbance in their maturation processes. The reticulocyte response to an acute anaemia produced by bleeding was the same in rats acutely poisoned by beryllium as in normal rats. The assumption is, therefore, that the changes in the circulating white cells are the result of damage to the other organs, and this is supported by the evidence of extensive infiltration of the liver and spleen with granulocytes which starts within a few hours of the injection of beryllium.

The Effect of Subcutaneous and Intraperitoneal Injections of Soluble Beryllium Salts.

The ordinary salts of beryllium—sulphate, chloride, lactate, oxyfluoride—are much less toxic when given by subcutaneous injection. At a dose level of 5 mg./kg. beryllium sulphate and lactate will not kill any mice. The same dose of

beryllium as oxyfluoride is fatal, but the pathological picture is not that of beryllium poisoning. Death is due to the toxic effects of the fluoride.

Both subcutaneous and intraperitoneal injections of beryllium salts give rise to a vigorous local reaction in the tissues, and a chronic inflammatory lesion will develop after a single subcutaneous injection. Histochemical staining shows that the beryllium becomes fixed to the tissues at the site of the injection, and in the rabbit many remain for as long as 3 months after an intradermal injection. The reaction takes place round this fixed beryllium. The salts of Ca, Mg, and Al do not produce any such local reactions.

If the beryllium is injected subcutaneously as a solution of sodium beryllium tartrate its toxicity is much greater than an equivalent dose of beryllium as lactate or sulphate. Thus at a dose of 5 mg./kg. no mice died after the subcutaneous injection of beryllium lactate or sulphate, while all mice died after injection of sodium beryllium tartrate. The pathological picture in these animals was characteristic of beryllium poisoning.

The beryllium in the sodium beryllium tartrate is not in an ionized form, and a much greater proportion gets carried away into the circulation. This was shown in an experiment with a series of mice injected with 200 μg. beryllium. Some received beryllium lactate and others sodium beryllium tartrate. Mice were killed at intervals after the injection and the liver, spleen and kidneys removed and examined spectrographically. Within 15 minutes there was the equivalent of 1000 parts beryllium per million parts of dry weight of liver, spleen and kidney in the animals receiving sodium beryllium tartrate, while no beryllium at all could be detected within 2 hours in the animals receiving beryllium lactate, and only traces were present 18 hours later.

Apparently ionic beryllium has the power of attaching itself rapidly and firmly to tissue protein. It also attaches itself to serum protein after intravenous injection. The histochemical staining always shows the beryllium to be present in amorphous masses within sinuses, etc., and these masses have all the other staining properties of protein.

If beryllium is added to serum *in vitro* it rapidly becomes firmly attached, and an ultrafiltrate of serum to which beryllium has been added in a concentration of 50 μg./ml. contains no beryllium at all. Ordinary salts of beryllium are precipitated by sodium phosphate, but if the beryllium is first added to serum and then sodium phosphate is added, no precipitate forms. Beryllium salts, such as the lactate, do not precipitate serum proteins *in vitro*.

Little is known about this peculiar affinity of beryllium for protein.

Although this attachment to protein will protect beryllium from precipitation as phosphate, it does not in any way reduce its toxicity. If beryllium lactate is first added to serum and left for periods up to 24 hours, this treated serum has all the toxic properties of the added beryllium.

DISCUSSION.

This peculiar affinity of beryllium for protein suggested the possibility that the syndrome of beryllium poisoning might be attributable to the destruction or inhibition of some specific enzyme as a result of beryllium becoming attached thereto.

Early in the course of the work it was discovered that rabbits died from beryllium poisoning with blood sugar levels of 20 mg./100 ml. or less. The

terminal convulsions were almost certainly hypoglycaemic in nature. Rats also died with low blood sugar levels.

It is hoped to report the full biochemical changes elsewhere, but it can be stated here that, as yet, no evidence has been found of any specific disturbance of the carbohydrate metabolism in the early stages of beryllium poisoning. Blood sugar levels, sugar tolerance curves, liver glycogen levels and blood lactic acid levels and tolerance curves were all within normal limits in the first 24 hours of poisoning. Disturbances did occur, but only at a time when they could be accounted for by the amount of the destruction of liver tissue that had taken place. The animals apparently die from an acute liver failure. This belief is supported by the experimental proof that repeated intravenous injections of glucose will prolong the life of fatally poisoned animals.

The destruction of the liver is apparently the result of an accumulation of beryllium, in the sinusoids which takes place within the first few hours of the intravenous injection of beryllium. Foci of necrosis start round the deposited beryllium and extend by enlargement and confluence to form a midzonal necrosis. Opie (1925), from a study of man and animals, concluded that focal necrosis was the antecedent condition for the development of midzonal necrosis. This same train of events has been seen after poisoning with a wide variety of agents—bacteria, bacterial toxins, vegetable toxins and metallic salts. As a rule the focal necrosis and subsequent zonal necrosis is centrilobular rather than midzonal. At first sight it appears paradoxical that the liver should accumulate within its sinusoids a substance such as beryllium,which then proceeds to destroy the liver tissue and so kill the animal. This appears less peculiar if it is assumed that beryllium, by attaching itself to protein, produces some foreign colloidal material. Foreign material of a collodial or particulate nature is normally segregated in the sinusoids of the liver and spleen, while hardly any can be found in the kidney, stomach, intestines, central nervous system or voluntary muscles (Drinker and Shaw, 1921 ; Lund, Shaw and Drinker, 1921, Drinker ; Shaw and Drinker, 1923; Voigt, 1914*a*, 1914*b*, 1915, 1918; Duhamel, 1919; Gye and Purdy, 1922).

Once these materials have been removed from the general circulation and collected in the liver sinusoids they are either taken up by the Kupffer cells or absorbed by the liver cells. It is presumably the attempt to absorb the beryllium that damages the hepatic cells. The diversity of agents that produce liver disease has led to a search for a final common path through which they exert their toxic effect. Though it has been suggested that some susceptible enzyme system might supply this path, no such system has yet been identified. An alternative hypothesis is that masses of foreign material in the liver sinusoids produce focal necrosis as a result of vascular stasis. Though this view has the authority of Mallory, the condition of stasis has yet to be adequately demonstrated. In the case of beryllium poisoning the first change seems to be an alteration in the cell permeability, resulting in cellular oedema. Foci of cells close to aggregates of beryllium then break down, and necrosis has started. This suggests that beryllium may affect the permeability of the cell membrane.

The lesions in the spleen apparently have an origin similar to those of the liver, for the cells that first show evidence of damage are those around the focal concentrations of beryllium in the sinuses of the marginal zone and in the red pulp. Necrosis and lysis spread until all but the reticulum itself is destroyed.

The cells of the hepatic parenchyma and those of the red pulp of the spleen bear no functional resemblance to one another. This suggests that the action of beryllium is a general one, and at the present time little more can be said than that beryllium or beryllium protein complexes irreversibly damage some system common to the surfaces of certain types of cell.

The renal lesions are not so readily accounted for. After large doses of beryllium intravenously the metal can only be detected in the *vasae recti*, and none can be seen within or around the tubules that subsequently show the damage. Chemical analysis shows that very little beryllium can be found in the whole kidney, and very little beryllium is excreted in the urine. However, before dismissing the possibility of direct damage to the tubules by beryllium, it must be remembered that no beryllium can be detected histochemically even in the liver after a single lethal dose. In other words, liver necrosis can result from the presence of amounts of beryllium too small to be detected histochemically. It is possible that in the first few minutes after injection some tubules come into direct contact with beryllium, but that the kidney reacts in a different way to the presence of this irritant. The liver collects the beryllium, but the kidney, possibly by means of the vascular shunt described by Trueta, Barclay, Daniel, Franklin and Prichard (1947), protects itself from further damage and beryllium has no means of accumulating within it. The occasional case where cortical necrosis develops may be the result of an overaction of this shunt.

Work with beryllium *in vitro* may often be complicated by the formation of the very insoluble beryllium phosphate. Although there is theoretically enough phosphate in the circulating blood to precipitate the beryllium injected as a single lethal dose, such precipitation and inactivation does not take place, presumably because of the protective role of the protein. On the other hand, when beryllium is given by mouth the inorganic phosphate in the gut is able to prevent the absorption of any significant quantity of beryllium. As Branion, Kay and Guyatt (1931) showed, feeding beryllium to rats will produce rickets by depriving the animals of phosphate, and these animals die with a gross phosphate deficiency. We have confirmed these observations and produced beryllium rickets, but rats dying during these experiments show none of the tissue changes that are characteristic of beryllium poisoning. Furthermore, the tissues of these animals, when examined spectrographically after 3 months on a diet containing 2·5 per cent beryllium as a soluble salt, contain only traces of the metal.

CONCLUSIONS.

Beryllium ions have the property of reacting rapidly with certain tissue proteins, including plasma protein. The complex so formed is of an unknown nature. It is of sufficient stability to protect beryllium from precipitation by phosphate ions. Such precipitation is the fate of beryllium ions reaching the alimentary canal, with the result that beryllium leaves the body without harming it except in so far as it may deprive it of phosphates where the amount of beryllium is large and its administration is continued for long periods.

The beryllium-protein complex is probably well held together because of the property beryllium possesses of forming co-valent compounds. The protein is not obviously denatured (precipitated) by the union with beryllium. At the same time, the original toxicity of the beryllium ions is fully maintained.

In the subcutaneous tissues beryllium attaches itself to protein forming part of the permanent tissue elements, for after a single injection beryllium may remain *in situ* for many weeks. Round the beryllium chronic inflammatory lesions develop and persist presumably as a response to the continual slight tissue destruction produced by the presence of beryllium. Because of this affinity for the fixed structures, beryllium injected into tissues or inhaled tends to remain localized. After a dose is administered in either of these ways the animal may die from acute beryllium poisoning if enough beryllium escapes from the site of injection to overwhelm the liver. In other cases the animal may die from the acute inflammatory effects produced by the beryllium, as, for example, when a large dose is inhaled and a secondary pneumonia develops.

When beryllium ions are injected into the circulation, they attach themselves to plasma protein and not to the endothelial lining of the vessel walls. The beryllium-protein complex is then removed from circulation by the liver, and by doing this the liver sows the seeds of its own destruction, for before it can excrete the beryllium, the latter has started to destroy the hepatic cells. Where the dose is large enough destruction may proceed almost to completion, but it can be halted after the injection of sublethal doses.

The exact nature of the change produced by beryllium on liver cells is unknown. It interferes with some intimate cellular mechanism, and other cells, such as the lymphocytes of the spleen, are also killed by beryllium. Vascular endothelium and neutrophil leucocytes appear to be more resistant to the action of beryllium.

Beryllium ions are poisonous to all living cells when brought into intimate contact with them. Their affinity for tissue protein and their insolubility in the presence of phosphate means that their toxicity to the whole animal is determined to a very large extent by the route of their administration.

SUMMARY.

1. The lethal dose of beryllium injected intravenously into rabbits, rats and mice as solutions of ionized salts is 0·5 to 1 mg. per kg. (20 micromol.).

2. Death takes place 1 to 3 days later and is the result of acute liver necrosis.

3. Neither the toxic properties of the anionic component nor the acidity of the solutions of the beryllium salts are responsible for these deaths.

4. By histochemical methods it is possible to see beryllium collecting in the liver sinusoids and initiating the necrotic changes.

5. Beryllium ions rapidly attach themselves to protein, and in this form are carried to the liver after intravenous injection. After subcutaneous injection, on the other hand, they tend to remain for long periods attached to tissue proteins.

6. Beryllium salts produce inflammatory changes in tissues after injection or inhalation, but as most of the beryllium is fixed to the tissues, death from liver necrosis only follows the injection of very large doses.

We would like to express our thanks to Dr. G. L. Miller, Chief Chemist of Murex, Ltd., for generous supplies of beryllium salts and for the care he gave to our special requirements.

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SOME EFFECTS OF ANTERIOR PITUITARY EXTRACT ON LIVER METABOLISM.*

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BURN AND LING (1930) were the first workers to note the effect of injections of extracts of the anterior lobe of the pituitary in producing a ketonuria in the rat. Since that time much work has been done on various aspects of this finding, but little on the effects of such extracts on liver metabolism. On evidence of an indirect nature, Collip, Kutz, Long, Thomson, Toby and Selye (1935) and Mirsky (1936) have concluded that the ketogenic fraction of such extracts produces its effects by acting solely on the liver. Shipley (1944) found that the addition of a crude extract of the anterior lobe to rat liver slices incubated in serum produced a 46 per cent and a 27 per cent increase in ketone body production in the case of the fed and fasted animal respectively. No experiments appear to have been done prior to 1941 on the livers of animals treated with such extracts. Livers of animals so treated are characterized by an intense deposition of fat, and it thus seemed worthwhile to examine their metabolic behaviour.

* This communication forms part of a thesis submitted to the University of Melbourne in 1943.

EXPERIMENTAL.

Animals.

Well-fed male guinea-pigs of about 500 g. body-weight were used throughout.

Anterior Pituitary Extracts.

Fresh beef anterior lobes were finely minced and ground with saline at room temperature for one hour. The extracts were then centrifuged and stored at +1° until required. No extract was kept longer than two days. The volume of the extract was so adjusted that 1 ml. was equivalent to 0·5 g. of fresh gland.

Animal treatment.

All animals injected with anterior pituitary extract were given either 1·0 or 3·0 ml. subcutaneously over the dorsal region, and were sacrificed at varying time intervals after injection.

Manometric and analytical techniques.

The methods followed were as described in an earlier communication (Ennor, 1942).

RESULTS.

The behaviour of livers from untreated guinea-pigs is illustrated by the results in Table I.

These results have, for the most part, been published in the earlier communication (Ennor, 1942), and are reproduced here for the purpose of comparison with those obtained in fatty livers, which are given in Table II.

TABLE I.—*Oxygen Consumption and Acetoacetate Production in Livers of Normal, Well-fed Guinea-pigs.*

Liver fat (g.%).	Control—no substrate.				Sodium butyrate—0·01 M.			
	Q_{O_2}.	$Q_{ac.}$	$Q^c_{O_2}$.	$Q^c_{ac.}$	Q_{O_2}.	$Q_{ac.}$	$Q^c_{O_2}$.	$Q^c_{ac.}$
5·4	6·1	0·2	7·6	0·2	6·6	1·6	8·3	2·0
6·5	5·0	0·2	6·7	0·2	7·4	1·2	9·9	1·6
5·3	5·1	0·1	6·4	0·1	7·9	1·4	9·9	1·8
6·8	3·7	0·1	5·0	0·1	6·2	1·5	8·2	2·0
6·5	4·9	0·1	6·4	0·1	8·8	2·7	11·6	3·6
6·4	3·9	0·0	4·7	0·0	7·8	1·2	9·5	1·5
6·5	4·3	0·2	5·6	0·3	8·1	1·8	10·5	2·4
5·3	6·6	—	8·3	—	11·6	—	14·5	—
8·2	4·4	0·0	5·9	0·0				
6·2	6·3	0·2	8·0	0·3				
6·6	4·4	0·0	5·7	0·0				
6·8	4·6	0·2	6·2	0·3				
6·9	6·2	0·0	8·4	0·0				

The quotients Q_{O_2} and Q_{ac} are defined, respectively, as the number of c.mm. of O_2 and number of c.mm. CO_2 (arising from acetoacetate)/mg. of dry weight of liver slice/hr.

The quotients $Q^c_{O_2}$ and Q^c_{ac} are defined, respectively, as the number of c.mm. of O_2 and number of c.mm. CO_2 (arising from acetoacetate)/mg. of *fat-free* dry weight of liver slice/hr.

TABLE II.—*Oxygen Consumption and Acetoacetate Production in Livers from Guinea-pigs Treated with Anterior Pituitary Extract (A.P.E.).*

Time after injection (hr.).	Dose A.P.E. g. fresh gland.	Liver fat (g. %).	Control—no substrate.				Sodium butyrate—0·01 M.			
			Q_{O_2}.	$Q_{ac.}$	$Q^c_{O_2}$.	$Q^c_{ac.}$	Q_{O_2}.	$Q_{ac.}$	$Q^c_{O_2}$.	$Q^c_{ac.}$
3	1·5	6·9	4·4	0	5·7	0	10·7	2·3	13·7	3·0
3	1·5	4·7	5·2	0·3	7·3	0·4	9·6	1·9	11·6	2·3
3	1·5	9·4	3·4	0·4	4·8	0·5	7·0	2·2	10·4	3·2
4	1·5	7·5	3·9	0·1	5·7	0·2	9·5	2·5	12·5	3·3
5	1·5	10·4	5·8	0·7	8·6	1·0	9·5	3·1	14·3	4·6
5	0·5	8·1	4·5	0·2	6·2	0·3				
5	0·5	6·9	3·7	0·1	4·9	0·2				
10	0·5	7·4	5·4	0·4	7·2	0·5	11·6	3·1	15·4	4·1
11	0·5	15·5	4·6	0·4	9·2	0·7	9·7	2·5	19·4	5·0
11	1·5	10·3	4·3	0·2	6·3	0·3	8·2	2·1	12·1	3·1
11	1·5	12·4	3·8	0·1	6·7	0·1	6·6	1·8	10·4	2·8
11	1·5	7·0	4·6	0·4	6·1	0·5	10·4	2·9	14·0	3·8
17	0·5	9·1	5·7	0·2	7·8	0·3				
18	1·5	19·7	3·4	—	7·4	—	6·9	3·1	15·0	6·7
19	1·5	13·3	5·4	0·5	9·0	0·9	8·2	2·3	13·6	3·8
24	1·5	19·9	4·2	0·3	9·5	0·6	6·4	1·6	12·8	3·1
24	1·5	20·0	4·5	0·3	8·8	0·6	7·1	2·1	13·7	4·0
24	1·5	15·0	4·9	0·4	8·4	0·7	7·0	2·0	13·5	3·5
32	1·5	13·2	5·4	0·8	9·0	1·4	8·6	2·2	14·3	3·7

In the absence of added substrate (Table II) there is no obvious departure from normality as far as the Q_{O_2} values are concerned. The Q_{ac} values, however, show a definite increase over the comparable figures from Table I. When the results calculated on the basis of fat-free dry weight are considered, there is evidence of an increase in the $Q^c_{O_2}$, whilst the Q^c_{ac} values are, of course, increased to a still greater degree.

Unfortunately the experiments carried out at each time interval are not sufficiently numerous to establish the sequence of events within the liver in relation to time. Broadly, however, it may be said that the effects of anterior pituitary extract are detectable three hours after injection. Although the fat content is not unequivocally changed, the Q^c_{ac} values are definitely in excess of normal. This result would seem to suggest that the first effect of the anterior pituitary extract is to increase the rate of ketogenesis, and thus to lend support to Shipley's (1944) conclusion (*cf.* Best and Campbell (1938)). As the time interval between injection and sacrifice is increased, the effect of anterior pituitary extract on liver fat and $Q^c_{O_2}$ and Q^c_{ac} becomes more pronounced. Consistently maximal figures for these are to be seen at the 24-hour interval, which was the longest period investigated.

There is also a difference in the behaviour of the livers from Table I and Table II in their reactions towards added substrate. These differences become apparent in Table III, where the difference between the quotients (expressed on a fat-free dry weight basis) for the oxygen consumption and acetoacetate production in the absence and presence of added substrate is shown.

TABLE III.—*Increments in Oxygen Consumption and Acetoacetate Production in Livers from Normal and Anterior Pituitary Extract-treated Guinea-pigs arising as a Result of Added Substrate.*

Normal.		Treated with anterior pituitary extract.	
$\Delta Q^c_{O_2}$.*	ΔQ^c_{ac}.†	$\Delta Q^c_{O_2}$.*	ΔQ^c_{ac}.*
0·7	1·8	8·0	3·0
3·2	1·4	4·3	1·9
3·5	1·7	5·6	2·7
3·2	1·9	6·8	3·1
5·2	3·5	5·7	3·6
4·8	1·5	8·2	3·6
4·9	2·1	10·2	4·3
6·2	—	5·8	2·8
		3·7	2·7
		7·9	3·3
		7·6	—
		4·6	2·9
		3·3	2·5
		4·9	3·4
		5·1	2·8
		5·3	2·3

* $\Delta Q^c_{O_2} = Q^c_{O_2}$ in presence of 0·01 M sodium butyrate—$Q^c_{O_2}$ in absence of substrate.

† $\Delta Q^c_{ac} = Q^c_{ac}$ in presence of 0·01 M sodium butyrate—Q^c_{ac} in absence of substrate.

There is evidence that, in the presence of 0·01 M sodium butyrate, the livers from the pituitary-treated animals show a greater oxygen uptake and a greater acetoacetate production than do the livers from the untreated animals.

In view of the general similarity of behaviour of the fatty liver arising from treatment with anterior pituitary extract to that following poisoning with carbon tetrachloride, it is of interest to compare the mean quotient values for oxygen consumption and acetoacetate production obtained from normal anterior pituitary extract-treated, and carbon tatrachloride-treated animals. Table IV illustrates the results obtained.

TABLE IV.—*Mean Values for Quotients Obtained from Livers of Normal Anterior Pituitary Extract and CCl_4-treated Guinea-pigs in the Absence of Added Substrate.*

	Q_{O_2}	Q_{ac}	$Q^c_{O_2}$	Q^c_{ac}
Normal	5·0	0·1	6·5	0·1
Anterior pituitary extract (> 10% fat) .	4·6	0·4	8·3	0·7
CCl_4 (> 10% fat)	6·1	0·3	11·5	0·6

In this table the mean figure for the normal values has been calculated from the figures given in Table I, and that for anterior pituitary extract-treated animals from Table II. In the latter case, only those values obtained with livers with more than 10 per cent of fat have been selected for calculation of the means. The results for the carbon tetrachloride livers have been taken from

Tables II and III in a previous publication of the author (Ennor, 1942), and here again only the figures from those animals whose livers contained more than 10 per cent of fat have been included in the calculation of the means. This selection of results has been made to ensure that only those livers with fat content definitely in excess of the normal upper limit are included for comparison.

As far as the Q_{O_2} means are concerned, the values for the normal and the anterior pituitary extract-treated livers are substantially the same, and since these figures are based on the dry weight of the liver slices, it is clear that, as the fat content of the anterior pituitary extract livers is in excess of 10 per cent, the actual respiring tissue must be utilizing more oxygen than is the case in the normal liver. This is substantiated by the $Q^c_{O_2}$ figures, which show that the anterior pituitary extract-treated livers have a definitely higher oxygen consumption. In the case of those livers from animals treated with carbon tetrachloride, the oxygen consumption is increased irrespective of the method of calculation. Similar remarks may be applied to the acetoacetate production from both types of fatty livers. It is therefore concluded that an influx of fat into the liver, due either to treatment with carbon tetrachloride or anterior pituitary extract is followed by an increase in the oxygen consumption and the acetoacetate production. There does, however appear to be a difference between the behaviours of these two types of fatty liver. It will be noted from Table IV that these livers have very nearly the same capacity for production of ketones, but that there seems to be a real difference in their ability to consume oxygen. Thus, the livers from the animals treated with anterior pituitary extract consume only two-thirds of the oxygen that is consumed by the livers from animals treated with carbon tetrachloride.

DISCUSSION.

The results of this investigation are of interest, particularly in view of the recent work of Campbell and Davidson (1949). These workers have also observed the increase in oxygen consumption and acetoacetate production of the livers from animals injected with anterior pituitary extract. They concluded that there was evidence of a pituitary factor which caused enhanced activity of the fat metabolizing centres. Such a conclusion is in agreement with the results reported here, since there is evidence that the pituitary fatty liver responds to added substrate by consuming more oxygen and producing more acetoacetate than does the normal liver.

Oxygen is necessary for the oxidation of fat, and if this is being oxidized rapidly and in amounts greater than normal, as is indicated by the high Q^c_{ac} values in all animals having fatty livers, then oxygen is necessary in amounts greater than normal in proportion to the amounts of ketone bodies being produced. This generalization agrees with the conditions existing in the livers of animals treated with anterior pituitary extract and with carbon tetrachloride. It is only when the results obtained with these types of fatty livers are compared that a difference in the degree of response is obvious. In the case of those animals treated with anterior pituitary extract, the increase in oxygen uptake is not of the same magnitude as in the case of the carbon tetrachloride fatty liver, though the acetoacetate production is comparable. Since the oxygen uptake is equal to the sum of the oxygen necessary for the oxidation of fat, plus that necessary for the oxidation of other substrates, it would seem that, in the case of the pitui-

tary fatty liver, the extra amount of oxygen necessary for the oxidation of fat is obtained at the expense of that necessary for the oxidation of other substrates. The net result is that oxygen is made available for the oxidation of fat without a marked increase in the total uptake being apparent. Such an hypothesis suggests that the anterior pituitary extract, in addition to producing an increase in liver fat, may also inhibit other enzyme mechanisms dependent upon oxygen. There is no indication from the present work as to what, if any, mechanisms are inhibited, but that inhibition is possible is suggested by the work of Colowick, Cori and Slein (1947). On the other hand, it is possible that the difference may be due to an overall increase in metabolism from carbon tetrachloride-treated animals. It is hoped that work at present in progress in this laboratory may give further information on this problem.

SUMMARY.

1. The effects of injections of crude extracts of the anterior pituitary gland on the liver metabolism of the guinea-pig have been investigated.

2. Associated with the increase in liver fat of animals so treated, there is an increase in the oxygen consumption and acetoacetate production.

3. Comparison of the results with those obtained in the fatty liver following treatment with carbon tetrachloride indicates a difference in response which may be due to an inhibitory influence of the extracts on oxidative processes other than those concerned with fat metabolism.

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SEROLOGICAL PROTECTION AGAINST THE "FATTY LIVER"-PRODUCING EFFECT OF ANTERIOR PITUITARY EXTRACTS.*

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IT was noted by Best and Campbell (1938) that the injection of extracts of the anterior lobe of the pituitary gland into guinea-pigs was followed by a marked rise in liver fat in the first 24 hours. It was also noted that a further rise, although less marked, occurred in the next 24-hour period, but on the third day the liver fat actually decreased in spite of continued injection of the extract. No explanation of this finding was offered by these workers, and it occurred to the present authors that these experiments bore a close analogy to those which were described in a communication dealing with the action of antidiabetogenic sera (Ennor and Singer, 1941). It was thought that the probable explanation of Best and Campbell's findings was that the animals had developed antibodies to the fat-producing factor, and that, after the initial accumulation of fat had been oxidized or otherwise removed, the antibody reaction was sufficiently great to negate the activity of the extract.

This communication gives the results of investigations into this hypothesis.

EXPERIMENTAL.

Anterior pituitary extracts.

All extracts used were prepared as described by Ennor (1949).

Preparation of anti-serum.

Adult English hutch rabbits were used throughout. Each animal was given an intravenous injection of 1 ml. of the anterior pituitary extract daily for the first three days, and thereafter 1 ml. on every third day until such time as the antibody titre was considered sufficiently high. The animals were then bled with aseptic precautions, and after clotting the serum was separated and stored at $+1°$ for use. The titre of the serum was tested by complement fixation with anterior pituitary extract diluted 1/10, and at the time of use was 1/320. Prior to injection, antibodies specific to ox serum proteins were removed by precipitation with ox serum.

Manometric and analytical techniques.

The methods followed were as described in an earlier communication (Ennor, 1942).

* This communication forms part of a thesis submitted by one of us (A.H.E.) to the University of Melbourne in 1943.

Animals.

Well-fed male guinea-pigs of about 500 g. body-weight were used throughout.

RESULTS.

Control series.

Several control experiments were carried out to determine the effect of normal rabbit serum and immune serum. In the case of those animals receiving serum, 6·0 ml. were administered intraperitoneally 18 hours before sacrifice. Such injections apparently exert no influence either on the fat content or on the reaction towards added substrate (Table I).

TABLE I.—*Oxygen Consumption and Acetoacetate Production of Livers from Guinea-pigs Untreated and Treated with Normal Serum and Immune Serum.*

Liver fat (g. %).	Control—no substrate.		Na butyrate—0·01 M.	
	$Q^{*}_{O_2}$.	$Q^{c}_{ac.}$ *	$Q^{c}_{O_2}$. *	$Q^{c}_{ac.}$ *
Normal controls.				
5·4	5·8	0·2	13·2	3·1
6·4	4·7	0	9·5	1·5
5·7	7·4	0·2	12·8	3·6
Normal serum controls.				
5·4	6·8	0·1	11·1	2·7
5·8	8·2	0	—	—
4·5	8·0	0	12·1	3·2
Immune serum controls.				
6·0	8·0	0·5	14·4	3·7
3·4	6·6	0·2	13·1	3·1

* The quotients $Q^{c}_{O_2}$ and Q^{c}_{ac} are defined, respectively, as the number of c.mm. of O_2 and number of c.mm. CO_2 (arising from acetoacetate) /mg. of fat-free dry weight of liver slice/hr.

Anterior pituitary extract treated series.

Here the animals were given a single subcutaneous injection of anterior pituitary extract which was equivalent to 1·5 g. of fresh gland. In the case of animals receiving serum in addition to the anterior pituitary extract, this was given 8 hours prior to the extract. The animals were then sacrificed as before, making a total of 18 hours' survival after injection of serum.

The results are shown in Table II, where " absorbed serum " refers to immune serum from which antibodies were removed by absorption with anterior pituitary extract before injection. As is well known, the injection of anterior pituitary extract is followed by an increase in liver fat, and this is uninfluenced by the injection of normal serum prior to the anterior pituitary extract. The metabolic behaviour of the livers from the two groups is similar. If, however, immune serum is substituted for normal serum, the injection of anterior pituitary extract does not result in any change of the liver fat content; complete protection is thus afforded. The oxygen consumption appears to be rather larger than normal, and the acetoacetate production definitely so. An explanation of this effect

TABLE II.—*Oxygen Consumption and Acetoacetate Production of Livers from Guinea-pigs Treated with Anterior Pituitary Extract and Anterior Pituitary Extract + Serum.*

Liver fat (g. %).	Control—no substrate.		Na butyrate—0·01 M.	
	$Q^c_{O_2}$ *	$Q^c_{ac.}$ *	$Q^c_{O_2}$ *	$Q^c_{ac.}$ *
Anterior pituitary extract only.				
15·4	9·2	0·7	19·4	5·0
10·3	6·3	0·3	12·1	3·1
Anterior pituitary extract + normal serum.				
11·9	8·6	0·1	11·1	2·7
10·6	7·6	0·4	12·1	2·3
Anterior pituitary extract + immune serum.				
6·3	6·4	0·2		
6·3	7·0	0·3		
6·0	6·9	0·4	12·4	3·0
5·7	6·5	0·3	13·0	3·4
6·5	6·8	0·2	12·7	2·8
7·0	8·4	0·6	13·7	3·8
Anterior pituitary extract + absorbed serum.				
13·2	11·1	—	11·3	3·1

* The quotients $Q^c_{O_2}$ and Q^c_{ac} are defined, respectively, as the number of c.mm. of O_2 and number of c.mm. CO_2 (arising from acetoacetate) /mg. of fat-free dry weight of liver slice/hr.

will have to await further work on the problem. The single experiment at the foot of Table II indicates that the protection gained by the prior injection of immune serum is completely lost if the serum is absorbed with crude extract before injection.

SUMMARY.

It is possible to inhibit the transference of fat from body depots to the liver, which follows subcutaneous injection of anterior pituitary extract in guinea-pigs, by prior administration of immune serum.

Part of the expenses of this work was defrayed by a grant from the Australian National Health and Medical Research Council to one of us (A. H. E.), and the work was carried out at the Baker Institute of Medical Research, Alfred Hospital, Melbourne.

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THE ISOLATION OF ANTIBIOTICS PRODUCED BY *PROACTINOMYCES (NOCARDIA) GARDNERI*.

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GARDNER AND CHAIN (1942) described some of the properties of a basic antibiotic produced by a chance contaminant on an agar plate. The organism was identified as a *Proactinomyces* by Waksman, and it was given the name *Proactinomyces (Nocardia) gardneri*. The antibiotic was called proactinomycin.

McKegney and Kent (unpublished, 1945) found that in surface culture the best yield of the antibiotic was obtained when the organism was grown on a potato dextrose medium containing 0·25 per cent agar. The extraction process of Gardner and Chain, which utilized the fact that the free base was more soluble than its salts in organic solvents, was modified by Abraham and Kent (unpublished, 1945) for large scale work.

The crude product was purified by partition between dilute hydrochloric acid and butyl alcohol-benzene mixtures on a silica gel column (Philpot, unpublished, 1946). No inhomogeneity was detected in the product obtained from the column.

Gardner and Chain found that the active substance could be precipitated from aqueous solution by base precipitants such as picric acid, picrolonic acid, flavianic acid, Reinicke acid and helianthic acid. The free base was slightly soluble in water and soluble in alcohol, acetone, ether, chloroform and benzene, and its salts with mineral acids were soluble in water, alcohol and acetone. Proactinomycin was stable in aqueous solution between pH 2 and 8, but was inactivated at room temperature at a pH greater than 10, or by boiling at pH 2. Philpot (unpublished, 1946) detected one titratable group with an approximate pK of 9. On mild treatment of proactinomycin with alkali she obtained (1) dimethylamine, (2) a base with an empirical formula $C_{18}H_{29}NO_5$, and (3) a neutral fraction with empirical formula $C_6H_9O_2$ from which an osazone could be obtained.

Heatley (unpublished, 1948) demonstrated in two counter-current distribution experiments that the product used in previous experiments was composed of at least three antibacterial components. This discovery prompted a reinvestigation of the properties of proactinomycin.

EXPERIMENTAL.

Production of material.

An attempt was made to select a high-yielding strain of the organism, and 11 single-spore isolates were tested by the cross streak method against *Staph. aureus*. No significant difference in the dimensions of the inhibitory zones was noted.

Stationary culture.—Part of the proactinomycin for these experiments was

prepared by the method of McKegney and Kent (unpublished, 1945) in rectangular ceramic vessels holding about 1 litre (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941) which were incubated at 24° C. for 9 days, at which time maximum activity was present, as measured by the cylinder-plate assay with *Staph. aureus* as the test organism (Heatley, 1944). The pH of the medium remained between 6·8 and 7·2, and the slight inconsistent changes in it could not be correlated with the level of activity. The medium was harvested after 9 days and squeezed through muslin or lint to remove the mycelium and break up the agar into small pieces.

Deep culture.—A preliminary experiment showed that maximum antibacterial activity was produced in aerated culture after a shorter period of incubation than was necessary for stationary cultures, although the final level obtained by either method was approximately the same. The medium was the same as that used for stationary culture except that no agar was included.

Some proactinomycin was prepared in 10-litre bottles set up as described by Stansly, Schlosser, Ananenko and Cook (1948), air being passed through at the rate of 1 litre per litre of medium per minute. The material was harvested after 5 to 7 days and yielded approximately the same quantity of crude active material as was obtained by the stationary culture method, i.e. about 80 mg. per litre.

Extraction and concentration.

The extraction and concentration process used was that of Abraham and Kent (unpublished, 1945):

> "The liquid was brought to pH 10, then shaken with an equal volume of amyl acetate. After settling, the amyl acetate was removed and shaken with three-tenths of its volume of acetate buffer at pH 4. The aqueous extract was brought to pH 10 and was again shaken three times with one-third of its volume of amyl acetate which was then concentrated by distillation *in vacuo* to one-sixth of its volume. This concentrate was shaken three times with one-tenth of its volume of water, the pH of which was maintained at about 4 by the addition of HCl. After concentration by vacuum distillation, the watery extract was dried by the lyophil process."

Separation of active substances.

The counter-current distribution method developed by Craig and others was used to separate and characterize three antibacterially active substances from the crude product. The procedure and theory of this method had been fully discussed in several papers (Craig, 1943, 1944; Craig, Golumbic, Mighton and Titus, 1945; Williamson and Craig, 1947; Barry, Sato and Craig, 1948), and they will not be considered here except in so far as required to make clear procedures which have been used and interpretations which have been made during the course of this work. A total of 25 counter-current distributions were performed.

The separation of a mixture of substances by the counter-current method depends on the relative distribution of the components between two immiscible liquids. The advantages of using a system in which the substance to be investigated is equally or nearly equally distributed between the two layers have been stressed by Craig and others. A satisfactory partition of the active principles

of proactinomycin between diethyl ether and M/2 phosphate buffer was easily accomplished. In the early procedures a mixture of Na_2HPO_4 and KH_2PO_4 were used, but later K_2HPO_4 was substituted for the Na salt because the latter tended to crystallize.

Eight tube distributions.—These were carried out in 600 ml. separating funnels, numbered 0 to 8. The two phases consisted of 200 ml. quantities of mutually saturated ether and buffer, the lower layer being transferred. Equilibrium between the two phases was ensured by 100 inversions at each step. At the end of the distribution the solute in each funnel was completely transferred to the ether layer by adjusting the pH to 10 with 40 per cent NaOH and adding enough NaCl to give a saturated aqueous solution. The volume of each ether layer was then adjusted to 200 ml. and transferred to a numbered bottle.

Aliquots of the ether solutions were used for dry weight and activity determinations. For dry weight determinations one of two types of container was used, depending on the volume of the aliquot. For 1 or 2 ml. aliquots, small aluminium cups weighing from 1·0 to 1·2 g. were used. The lightest cup in the group was used as a counterpoise. For larger aliquots thin glass tubes were used, and with these the ether was evaporated by placing the tubes under a stream of air. The cups or tubes were then dried to constant weight *in vacuo* over P_2O_5. The total weight of the solute in the system was determined by totalling the dry weights per funnel and this was used in calculating the percentages shown on the graphs. The weighing error was estimated as $\pm$ 0·1 mg. The weight of the aliquot taken from the peak tube in most procedures exceeded the estimated error at least 40–50 times, and in one (Fig. 6) 150 times.

Assays of antibiotic activity were carried out by shaking an aliquot of each ether solution with M/15 phosphate buffer, pH 7·0, and evaporating the ether. The buffer extracts were assayed by the cylinder-plate method and the percentage of the total activity in each determined by a comparison of the zones of inhibition with those of an aliquot of the starting solution, suitably diluted and assayed on the same plates. (In some cases, when only a rough determination of the activity was desired, filter paper discs cut with a cork borer were dipped in the ether solutions for the count of 5, dried by waving in the air for the count of 15, and placed on seeded plates (Sherwood, Falco, and de Beer, 1944).)

Twenty-four tube procedures.—Twenty-four tube distributions were carried out in an apparatus constructed at the Rockefeller Institute for Medical Research which is similar to that described by Craig (1944) except that it contains 25 tubes instead of the 20 of the earlier model. A small electric motor rotated the apparatus at the rate of 20 r.p.m., 50 revolutions (counted on a bicycle cyclometer) being used for each mixing, with a 5-minute period for the separation of the two layers before the next transfer.

After completion of the 24 transfers the threaded metal plug in the top section of the apparatus was replaced by a cork carrying a short length of glass tubing. By inverting the apparatus the contents of any tube could be emptied through this directly into a bottle with only a small loss of material. The organic solute in each tube was extracted into the ether layer by the addition of 40 per cent NaOH until the pH of the watery phase was 10. The layers were separated in a 20 ml. separating funnel and the buffer shaken with fresh ether. The two ether extracts were combined and the volume made up to 15 ml. Dry weight and activity determinations were carried out as in the 8-tube procedure.

RESULTS.

Gardner and Chain (1942) suggested that should additional substances of similar origin to proactinomycin be discovered " this agent herein described may be called Proactinomycin 1, the next Proactinomycin 2, and so on." As the substances investigated in this paper are active components of this supposedly single agent, they have been termed proactinomycin A, proactinomycin B and proactinomycin C until their chemical structure is known. An inspection of preliminary results suggested that the substance with the highest partition coefficient (hereafter called proactinomycin C or Pa C) might prove to be a single active substance and should be relatively easily separated. The separation of the components of the broad mixture in the centre of the curve was expected to offer some difficulties. The general plan adopted was to subject a sample of crude proactinomycin to an 8-tube funnel procedure and then to carry out a

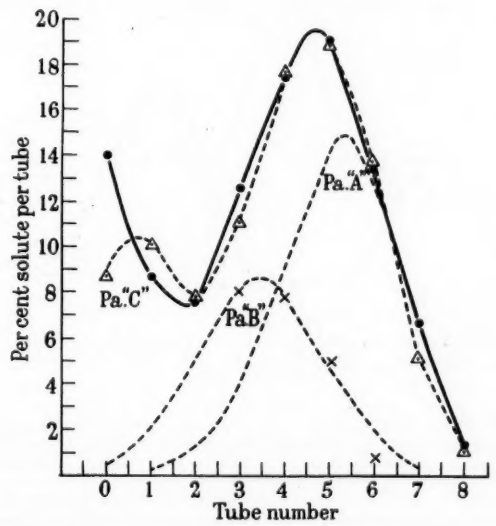

FIG. 1.—(8-tube funnel distribution, lower phase moving). pH of buffer, 7·0. The material used in this distribution was obtained from an aerated culture. The activity curve shows that at least two active substances were present in the starting material as two peaks are present, i.e. at Tube 1 and Tube 5. Furthermore, the portion of the curve from Tubes 3–8 could only be explained by assuming the presence of at least two active substances in some of these tubes. An attempt was therefore made to determine theoretical curves for two substances which, when summed, would coincide with the dry weight curve over these tubes.

The theoretical curves were derived as follows: Curve A is a curve expanded from the percentage of material in T_6 for a homogeneous substance having a K value of 0·55. The calculated experimental K for T_6–T_7 is 0·534, and for T_7–T_8 is 0·575. Curve B is a theoretical curve (K = 1·3) best fitting the points obtained by subtracting Curve A from the dry weight curve at T_3 to T_6. On the basis of these curves the starting material would have been composed of 50 per cent of A and about 30 per cent of B.

This interpretation is meant to be only approximate (note divergence in theoretical and dry weight curves at T_6), but Fig. 2 and 3 prove that it is very nearly correct.

——●—— Experimental. - - - -△- - - - Activity.
- - - - - - - - - - - Theoretical. × Experimental minus "A."

24-tube procedure on selected fractions rich in the substance having the lowest partition coefficient (termed practinomycin A or Pa A). A second 24-tube procedure would then be carried out on a mixture of substance Pa C and the substance proactinomycin B (or Pa B), having a partition coefficient intermediate between Pa A and Pa C. Fig. 1, 2 and 3 show the results of this approach on a sample of powder obtained from an aerated culture. It will be noted that the pH of the buffer in each procedure was chosen so that the best separation might be gained. In general, with the crude material the pH was 7·0, with Pa A 7·2, with Pa B or mixtures of Pa B and Pa C 6·8, and with Pa C alone 6·5. The results shown in Fig. 1, 2 and 3 were encouraging, but other batches of material were not so satisfactory as they contained a larger percentage of inactive impurities;

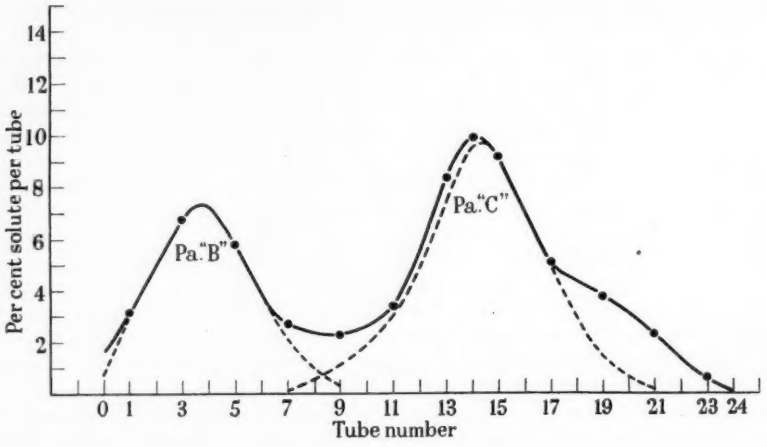

FIG. 2.—(24-tube machine distribution). pH of buffer, 6·5. The material used in this procedure was obtained by pooling the ether extracts from T_0, T_1 and T_2 of Fig. 1 with a small amount of material rich in Pa C obtained from previous 24-tube procedures. The pooled extracts were concentrated by vacuum distillation and water removed by freezing with a mixture of solid CO_2 and alcohol. The partition coefficient of the mixture of active substances was determined experimentally and found to be approximately 1 when the pH of the buffer was 6·5. In later procedures a slightly higher pH was used (pH 6·8) for mixtures of Pa B and Pa C so as to shift the curve slightly to the right.

The activity curve (not shown here) followed the theoretical curves very closely, there being no indication in this or any other distribution of any active substance in addition to the three under investigation. A relatively small amount of inactive impurity is present between Pa B and Pa C. (Compare with Fig. 4.)

——●—— Experimental. - - - - - - - - - - - Theoretical.

one of these was especially difficult to deal with as it exhibited a partition coefficient between that of Pa B and Pa C. The reason for this difference between different batches is not definitely known.

The graphs given in Fig. 1 to 6 have been selected from the 25 runs made to illustrate most clearly the kind of results obtained and the procedure adopted in certain cases.

Isolation of Proactinomycin A, B and C.

Proactinomycin A.—As already stated (Fig. 3), homogeneous or nearly homogeneous Pa A could be crystallized readily from a concentrated wet ether solution. The crystals formed as short, thick plates or short prisms. Recrystallization attempts with any of the solvent mixtures tried (ether or 40°–60° petroleum ether with chloroform, alcohol or acetone) were unsuccessful. However, crystals with a melting point of 168–169° C. (from T_9, Fig. 6) could be obtained from crystals contaminated with a small amount of impurity by first washing with ether, then dissolving the crystals in a small quantity of chloroform and adding ether to the chloroform solution. After melting point determinations, crystals of Pa A recrystallized on cooling. Attempts to crystallize Pa A from very impure solutions were not successful.

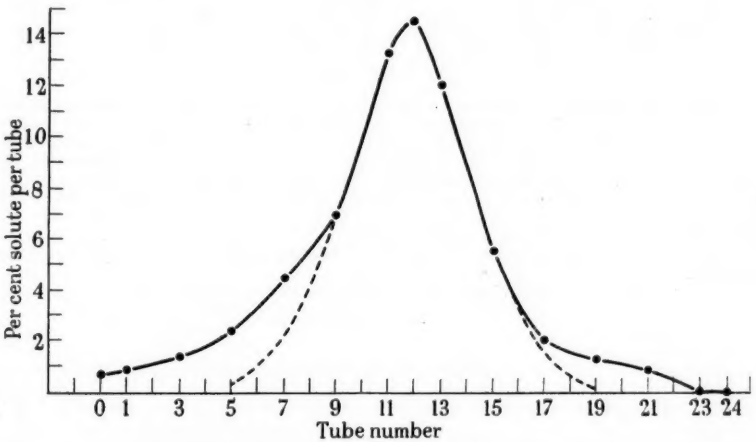

FIG. 3.—(24-tube machine distribution). pH of buffer, 7·2. The material used in this procedure was obtained from T_6, T_7 and T_8 of Fig. 1, to which was added a small amount of other material rich in Pa A from previous distributions. The ether solution was concentrated and the water removed as described under Fig. 2. This concentrated solution was left in the refrigerator overnight. Next morning many " button-like " groups of crystals (M.P. 165–167° C.) were present on the sides of the vessel. The mother liquor gave the distribution shown in this curve.

An aliquot of the ether solution from T_{11}, evaporated quickly under reduced pressure, yielded a clear, colourless, sticky oil which hardened on drying over P_2O_5. When a small amount of ether was added to the tube containing this oil, the fluid immediately became turbid, and in a few minutes crystals could be detected. These crystals were soluble in chloroform, acetone, alcohol and dilute acid, only slightly soluble in ether and light petroleum ether, and insoluble in water and dilute alkali. The melting point on the hot stage micro-melting point apparatus was 168–169° C.

——●—— Experimental. - - - - - - - - - - Theoretical.

Proactinomycin B.—A doubtfully crystalline solid could be obtained from pure Pa B by adding light petroleum ether to the homogeneous free base obtained as an oil after evaporation of an ether solution. These " crystals " melted at 83–87° C. on the hot stage micro-melting point apparatus.

Proactinomycin C.—Attempts to crystallize this substance were not successful.

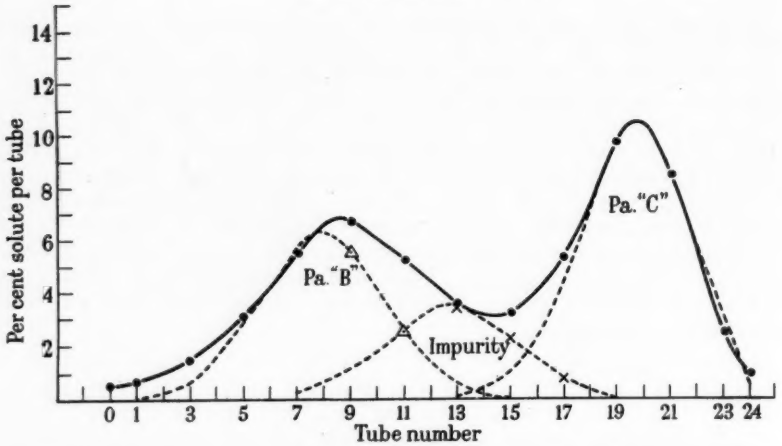

FIG. 4.—(24-tube machine distribution). pH of buffer, 6·8. This curve is presented to show the presence of a homogeneous inactive substance with a partition coefficient between that of Pa B and Pa C. The inconvenience which this impurity caused from a preparative standpoint, when present in relatively large amounts, is seen from a comparison of this curve with Fig. 2. In the latter at least four tubes containing the largest amount of Pa B were selected as being homogeneous within the limits of the method, whereas in the present case, none of the tubes were used as a source of material for experiments on Pa B until additional distributions had been performed.

The yield of homogeneous Pa C was also markedly reduced by the presence of this impurity.

——●—— Experimental. × Experimental minus "C."
- - - - - - - - - - - Theoretical. △ Experimental minus "Impurity."

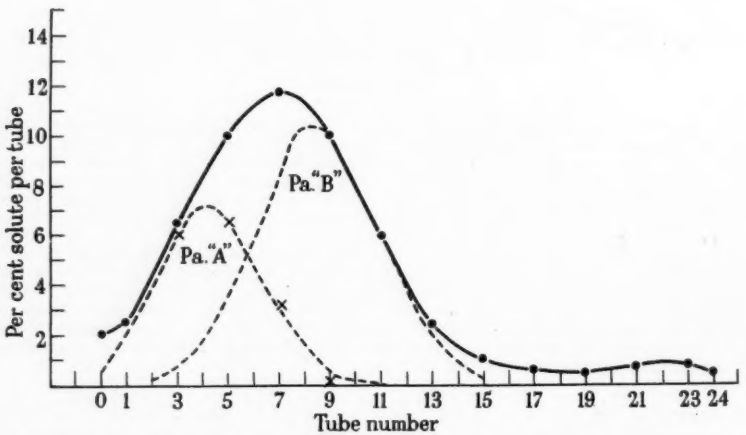

FIG. 5.—(24-tube machine distribution). pH of buffer, 6·8. This curve shows that when Pa A and Pa B were both present, little separation was obtained in 24 transfers. K for Pa A = 0·23, and for Pa B = 0·56. The starting material was obtained from selected tubes from a 16-tube alternate withdrawal procedure (Craig, Hogeboom, Carpentor and du Vigneaud, 1947) in funnels.

——●—— Experimental. - - - - - - - - - - - Theoretical. × Experimental minus "B."

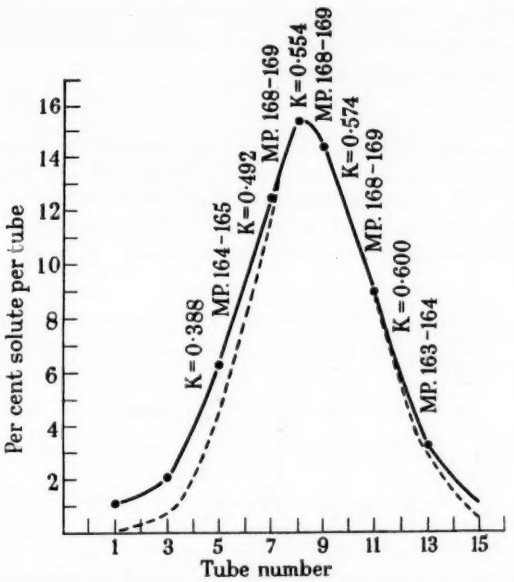

FIG. 6.—(24-tube machine distribution). pH of buffer, 7·2. This distribution was carried out on crystals of Pa A. Ten ml. aliquots were used for dry weight determinations and the melting points of crystals from five tubes were determined.

——●—— Experimental. - - - - - - - - - - Theoretical.

Chemical properties of proactinomycin A, B and C.

Chemical analyses (by Weiler and Strauss) of the three antibiotics gave the following results in percentages :

	Proactinomycin A.	Proactinomycin B.	Proactinomycin C.
C	63·2	63·8	65·5
H	9·04	9·4	9·2
N	2·67	2·71	3·08
O (by difference)	25·09	24·09	22·22
$(N)CH_3$	7·35	8·7	8·5
$(C)CH_3$	18·8	18·04	18·6
OCH_3	1·1	0	0
Molecular weight (Rast)	445	513	322

(The sulphur content of the three samples was 0·0096, 0·041 and 0·057 per cent respectively.)

On the basis of these figures the following molecular formulae are suggested :

Proactinomycin A, $C_{27}H_{47}O_8N$. (Requires C, 63·2 ; H, 9·2 ; N, 2·7.)
Proactinomycin B, $C_{28}H_{49}O_8N$. (Requires C, 63·8 ; H, 9·3 ; N, 2·7.)
Proactinomycin C, $C_{24}H_{41}O_6N$. (Requires C, 65·6 ; H, 9·3 ; N, 3·2.)

The analytical figures indicate that proactinomycin A and proactinomycin B are closely related compounds. The values for $(N)CH_3$ show that all three

antibiotics contain an $-N(CH_3)_2$ grouping. This is compatible with the finding of Philpot (unpublished, 1946) that dimethylamine was a product of the treatment of crude proactinomycin with alkali.

Ultraviolet spectra.

The ultraviolet absorption curves given by solutions of Pa A, Pa B and Pa C in anaesthetic ether are shown in Fig. 7. Measurements were made with a Beckman model DU photoelectric spectrophotometer and absorption due to solvent alone was automatically subtracted. The cell length was 1 cm. The measurements were made by Dr. V. E. Sharp and Dr. G. V. R. Born.

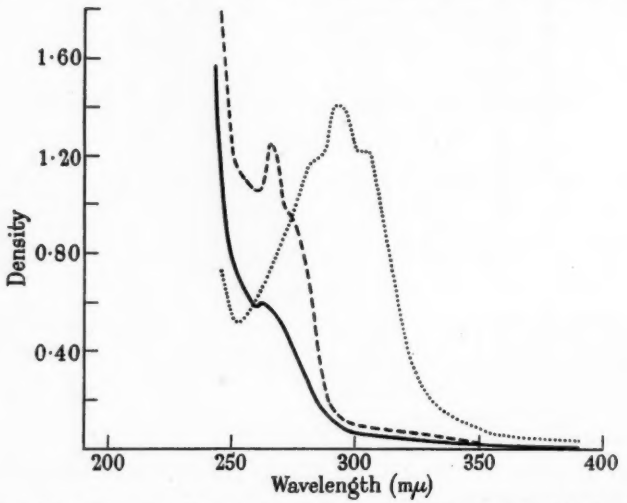

Fig. 7.—Ultraviolet absorption of proactinomycin A, B and C. Measured in the Beckman model DU spectrophotometer in anaesthetic ether. Thickness of layer, 1 cm.

———— Pa "A" 0·5 mg./c.c.
- - - - - - - - - - - Pa " B " 0·5 mg./c.c.
. . . . . Pa " C " 0·01 mg./c.c.

SUMMARY.

A reinvestigation of proactinomycin has resulted in the isolation of three basic antibiotics, one of which has been obtained in a crystalline form.

The work reported in this paper was carried out under the general supervision of Dr. N. G. Heatley. Mr. G. G. F. Newton has offered many helpful suggestions during the course of the work, and invaluable technical assistance was given by Mr. J. Kent and Miss S. Long.

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THE ANTIBACTERIAL AND PHARMACOLOGICAL PROPERTIES OF THE PROACTINOMYCINS A, B AND C.

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(*From the Sir William Dunn School of Pathology, Oxford.*)

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THE separation of three distinct proactinomycins from the antibacterial products of *Proactinomyces* (*Nocardia*) *gardneri* has been described (Marston, 1949). The present paper describes investigations of some of the antibacterial and pharmacological properties of these three substances, Proactinomycins A, B and C (called for convenience Pa A, Pa B and Pa C), and of the unfractionated substance, Pa crude.

EXPERIMENTAL.

Antibacterial Properties.

Methods.

The antibacterial titres of the preparations were ascertained by the serial dilution method. Two-fold serial dilutions in distilled water, pH 6·5, were sterilized by filtration through a sintered glass filter, and were kept in the refrigerator when not being used. The same batch of heart extract broth was used for all tests, but 10 per cent serum was added for *Str. pyogenes*, *Str. pneumoniae* and *C. diphtheriae*, and 2 per cent glucose in addition for *N. meningitidis*. Each tube containing 1·8 ml. of medium and 0·2 ml. of antibiotic solution was inoculated with one drop (delivered with a Dreyer's pipette) of an overnight culture of the organism (2 drops of *N. meningitidis*).

The tests were read after 24 and 48 hours of incubation at 37° C., but for the sake of clarity only the 48-hour results are given in the tables. In general the 24-hour readings showed inhibitory titres 2 to 4 times as high as those at 48 hours.

To ascertain the effect of serum on antibacterial activity 10 per cent of sterile horse serum was added to the medium in serial dilution tests. To ascertain whether whole blood impaired activity, the following procedure was adopted :

Five ml. of each preparation were made isotonic to horse red blood cells by the addition of 0·5 ml. of 9 per cent sodium chloride. To each of these solutions was added 4·5 ml. of oxalated whole blood, and the mixtures were rotated on a

tilted gramophone turntable (Heatley, 1941) for 4 hours at 37° C. They were then centrifuged and the clear supernatants, which were coloured by haemolyzed blood, were removed to four separate tubes which were placed in the refrigerator overnight and tested the following day by the serial dilution method against *Staph. aureus*.

Ten ml. of distilled water were added to the red cell pack (approximately 1 ml. in volume) remaining in each tube. This procedure readily lysed the cells, and these solutions were tested by the same method.

RESULTS.

Activity against various bacteria.

The results of the serial dilution tests are given in Table I. Three of the preparations had about the same activity, but Pa B was distinctly inferior against all the organisms tested except *N. meningitidis*.

In view of Abraham and Duthie's investigation (1946) of the effect of pH on the antibacterial activity of proactinomycin, the pH of the medium was determined at the end of two of the tests (*B. anthracis* and *Staph. aureus*). There was no difference in comparable tubes containing different preparations. The apparent inferiority of Pa B is not, therefore, to be explained by a change in pH.

TABLE I.—*Comparison of the Antibacterial Activity of the Four Preparations of Proactinomycin against Various Bacteria by the Serial Dilution Method. Figures show the Dilution of the Substance at the End-point indicated.*

Preparation.	Kind of inhibition.	*Staph. aureus.*	*Str. pyogenes.*	*Str. pneumoniae.*
Pa crude	Partial	1 : 40,000	1 : 640,000	1 : 1,280,000
	Complete	1 : 20,000	1 : 160,000	1 : 640,000
Pa A	Partial	1 : 40,000	1 : 640,000	1 : 1,280,000
	Complete	1 : 20,000	1 : 80,000	1 : 640,000
Pa B	Partial	1 : 10,000	1 : 160,000	1 : 640,000
	Complete	—	1 : 20,000	1 : 160,000
Pa C	Partial	1 : 80,000	1 : 640,000	1 : 1,280,000
	Complete	1 : 20,000	1 : 80,000	1 : 640,000

Preparation.	Kind of inhibition.	*B. anthracis.*	*C. diphtheriae* var. *gravis.*	*N. meningitidis.*
Pa crude	Partial		1 : 640,000	
	Complete	1 : 80,000	1 : 160,000	1 : 80,000
Pa A	Partial	1 : 80,000	1 : 320,000	
	Complete	1 : 40,000	1 : 160,000	1 : 80,000
Pa B	Partial	1 : 20,000	1 : 80,000	
	Complete	—	1 : 40,000	1 : 160,000
Pa C	Partial		1 : 1,280,000	
	Complete	1 : 80,000	1 : 320,000	1 : 40,000

A dash indicates that the figure was outside the range of the titration.
A blank indicates that there was no partial inhibition, as tubes showing no growth and full growth were adjacent in the dilution series.

The *B. anthracis* and *C. diphtheriae* var. *gravis* were laboratory stock strains. The other organisms were as follows: *Staph. aureus*, Wellcome CN 491; *Str. pyogenes*, Wellcome CN 10; *Str. pneumoniae*, Wellcome CN 33; and *N. meningitidis*, Colindale II 1615.

Effect of serum.

The presence of 10 per cent horse serum had no marked effect on the antibacterial activity of any of the four preparations against *Staph. aureus* (Wellcome CN 491).

Effect of whole blood.

Florey, Jennings and Sanders (1945) found that the activity of proactinomycin was reduced by incubation for 4 hours with undiluted whole blood. When this was repeated by the method described above there was an indication of slight loss of activity by all the substances, but residual activity was in all cases at least 50 per cent. Separate titration of the red cells after lysis showed an approximately equal distribution of the antibiotics between the red cells and the surrounding fluid.

Effect of size of inoculum.

The titre of all four preparations against *Staph. aureus* was only moderately affected by changes in the size of the inoculum. In each case a thousand-fold reduction in the density of the inoculum resulted in an increase in titre, usually about four-fold (Table II).

TABLE II.—*Effect of Inoculum Size on the Antibacterial Activity of the Four Preparations against* Staph. aureus. *Figures show the Dilution of Substance Completely Inhibiting Growth.*

Preparation.	Inoculum : overnight culture of *Staph. aureus.*		
	Undiluted.	Diluted 1 : 1000.	Diluted 1 : 1,000,000.
Pa crude	1 : 20,000	1 : 80,000	1 : 640,000
Pa A	1 : 20,000	1 : 80,000	1 : 160,000
Pa B	—	1 : 20,000	1 : 40,000
Pa C	1 : 20,000	1 : 80,000	1 : 320,000

Relation of titre by dilution test to zone size in cylinder-plate assays.

Under the conditions used (cylinder-plates surface seeded with a 1 : 100 dilution of a broth culture of *Staph. aureus*) a solution containing about 0·2 mg./ml. (1 in 5000) of any of the preparations gave a zone of inhibition about 20 mm. in diameter. The concentration-zone diameter curve obtained by the cylinder-plate assay was fairly steep, the zone diameter increasing by approximately 2 mm. for each doubling of concentration.

Pharmacological Properties.

Acute toxicity to mice.

One hundred and ten 20 g. mice in groups of from 5 to 8 were injected intravenously with various amounts of substances Pa A, Pa B, Pa C and Pa crude dissolved in 0·3 ml. of saline. The approximate LD_{50} of each substance was calculated by the method of Reed and Muench (1938), and was found to be for Pa A 3·0 mg., for Pa B 2·4 mg., for Pa C 1·6 mg., and for Pa crude 1·8 mg.

Some reaction was noted in all of the mice tested except those receiving

Pa A 1·8 mg. and Pa crude 1·3 mg. Symptoms began immediately after the injections, and ranged from a slightly shaky gait to violent convulsions followed by death in from 1 to 5 minutes. In some cases cyanosis was present. All of the mice which survived appeared normal 10 to 15 minutes after the injection, and from that time to the end of a 10-day observation period they presented no abnormalities.

Toxicity to leucocytes in vitro.

Effect on motility.—Tests in which an antibacterial substance is brought into direct contact with leucocytes *in vitro* give an opportunity for direct observation of the effect of the substance on living cells, and are also therapeutically important because of the essential role played by leucocytes in complementing the curative action of bacteriostatic substances.

The effects of the three substances Pa A, Pa B and Pa C on the motility of human leucocytes *in vitro* were compared by the method described by Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941). Each substance was tested in 9 dilutions ranging from 1 to 500 to 1 to 80,000. Some of the dilutions were tested more than once, and these duplicate tests gave consistent results. Pa A, which was the least toxic, killed leucocytes in 20 minutes at a concentration of 1 in 500, and impaired their motility at 1 in 10,000, but a concentration of 1 in 20,000 was without effect during an hour's observation. Pa B at 1 in 500 killed them more quickly, and at 1 in 25,000 impaired motility after an hour. Pa C was considerably more toxic than either of the others, as a concentration of 1 in 8000 killed the cells in 20 minutes, and 1 in 40,000 killed them in an hour.

Effect on phagocytosis.—Leucocytes were obtained from the peritoneal cavity of rabbits, after the injection of saline on two successive days, by the method described by Mudd, Lucké, McCutcheon and Strumia (1929). These were brought into contact with heat-killed staphylococci suspended in 5 per cent serum in saline containing different concentrations of the substances, by slow rotation at 37° C. for one hour. At the end of this time smears were made and fixed, and stained by Claudius' modification of Gram's stain, and the number of cells containing bacteria was counted.

There was no appreciable difference between the number of cells containing bacteria (about 80 per cent) in control preparations and in preparations containing concentrations of Pa A and Pa B as high as 1 : 2000. Phagocytosis in concentrations of 1 : 4000 to 1 : 8000 of Pa C was about the same, but in 1 : 2000 of this substance less than 20 per cent contained ingested bacteria.

Thus by both tests Pa C was more toxic than the other two substances to leucocytes, as it was to mice.

Effect on nerve tissue.—The toxicity of the substances to tissue cells was further tested by introduction into the cisternal space of rabbits, thus bringing them into direct contact with the central nervous system. The procedure was as follows : The rabbits were given atropine preoperatively and anaesthetized with ether. The spinal cord was entered by a cisternal puncture, and 0·5 ml. of cerebro-spinal fluid withdrawn and replaced by the same quantity of a saline solution (pH 6·5) containing from 1 to 10 mg. of the antibiotics. After recovery from the anaesthetic the animals were watched for signs of irritation of the central

nervous system. In most cases they were killed 24 hours after the injection, when a sample of the cerebro-spinal fluid was assayed for antibacterial activity and the brain stem was inspected.

In only one rabbit (after injection of 2 mg. of Pa B) was any gross damage to the nervous system, presumably due to the needle, found in the post-mortem examination. In this case haemorrhage had extended into the substance of the brain stem.

No symptoms which could be related to the effect of substances Pa B and Pa C were noted from maximum doses of 6 and 12 mg. respectively, but Pa A obviously had a detrimental effect when injected into the spinal canal. After-effects varied from post-operative " head shaking " when 3 mg. were injected, to death within 5 to 10 minutes after the injection of 10 mg. Six mg. in one rabbit caused marked symptoms, which began while the animal was still recovering from the anaesthetic, and consisted of, first, vigorous shaking of the head, then scratching motions and inability to stand. About 45 minutes after the injection it began to have fits every two or three minutes. These started with rapid, side-to-side motions of the head, and progressed quickly to violent, uncoordinated jerking of the whole body with a tendency to fall to the right.

The frequency and violence of the fits decreased rapidly during the next 30 minutes, and four or five hours after the injection the rabbit appeared quiet but otherwise fairly normal. After the seizures had become less frequent and less violent, stroking the head tended to bring on involuntary shaking of the head, but noise had no effect.

Symptoms similar to these, but less violent, were noted in one out of two rabbits given 10 mg. of Pa crude, but not in the other.

Antibacterial activity was detected in the cerebro-spinal fluid 24 hours after injection in animals that received as much as 3 mg. of Pa A, 2 mg. of Pa B, and 12 mg. of Pa C.

The most interesting feature of these results is that the order of toxicity to nerve tissue, as shown by these tests, differed from that to whole mice and to leucocytes *in vitro*. Pa A was the most toxic of the three substances to the central nervous system, and was the least toxic of the three in the other tests.

Effect on blood pressure and respiration of decerebrate cats.

Florey, Jennings and Sanders (1945) found that proactinomycin caused a rise in the blood pressure of cats under chloralose anaesthesia. A rise was obtained under nembutal anaesthesia, though on repeating the injection of the antibiotic a fall occurred. In the decerebrate cat there was a slow rise of pressure after an initial fall, while in the decapitated cat injection was followed by a sharp fall, with recovery up to but not above the original level.

In the present experiments the effects of Pa A, Pa B, Pa C and Pa crude on the blood pressure were investigated on six decerebrate cats, and Pa crude on one cat under chloralose anaesthesia. The method of decerebration was that described by Schmidt (1923), in which the brain is divided by drawing a ligature tight, so that bleeding is avoided.

The substances were dissolved in 5 to 8 ml. of saline, pH 6·5–7·0, and were administered slowly, taking two or three minutes, by intravenous injection. The results obtained in these experiments with Pa crude resembled those reported by Florey *et al.* with proactinomycin in the decerebrate cat in that the pressure

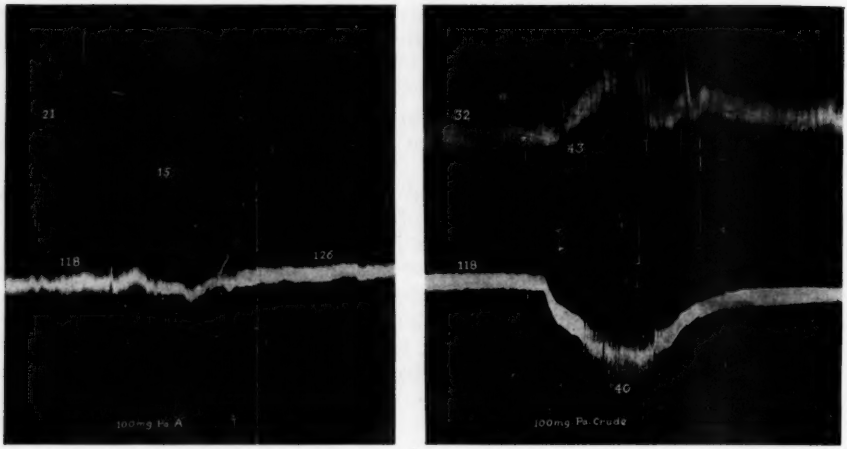

FIG. 1.—*Cat* M/4 (decerebrate). Weight 2·8 kg. (11-minute break between end of first and beginning of second tracing). The injection of 100 mg. of Pa A (36 mg. per kg.) caused a slight fall of blood pressure (10 mm. of Hg.) followed by a slight rise. The respiratory rate decreased from 21 to 15 r.p.m. during the injection. Thirty minutes after this injection 100 mg. of Pa crude (36 mg. per kg.) were injected into the same cat. This dose caused a pronounced fall in blood pressure, from 118 mm. to 40 mm. of mercury, and an increase in respiratory rate from 32 to 43 r.p.m. [The time signal in Fig. 1 to 5 indicates 10 seconds.]

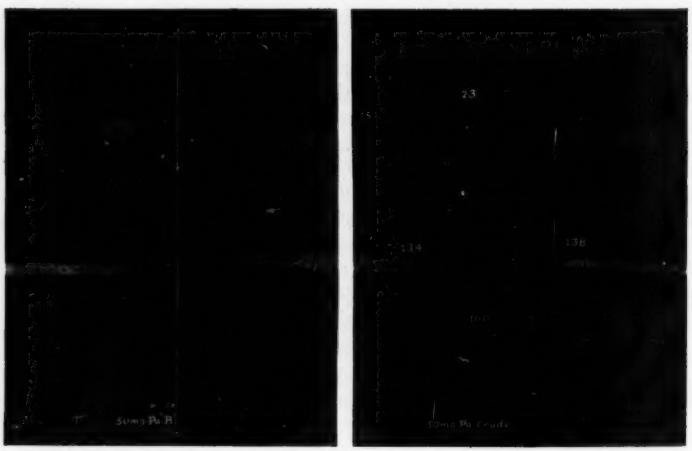

FIG. 2.—*Cat* M/3 (decerebrate). Weight 2·6 kg. (10-minute break between end of first and beginning of second tracing). An injection of 50 mg. of Pa B (19 mg. per kg.) had no immediate effect on the blood pressure, but a slight rise occurred, starting a few minutes after the injection was completed. There was no effect on the rate of respiration. Fifty mg. of Pa crude were injected 20 minutes after the first injection, and resulted in the marked fall in blood pressure and increased respiratory rate shown in the adjacent tracing.

fell and then rose again. In the present work, however, the initial fall was more pronounced, and though there was afterwards recovery, the original level was only exceeded in a few animals. In the one cat tested under chloralose

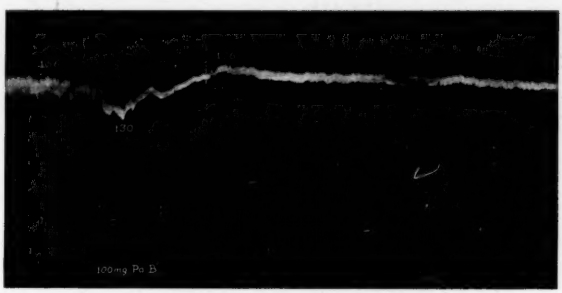

FIG. 3.—*Cat* M/7 (decerebrate). Weight, 2·8 kg. One hundred mg. of Pa B, or 36 mg. per kg., caused a fall in blood pressure followed by the most pronounced rise (20 mm. of mercury) encountered in these experiments.

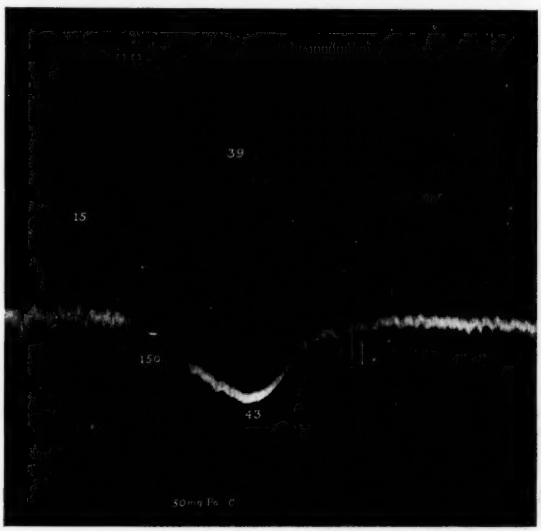

FIG. 4.—*Cat* M/5 (decerebrate). Weight 2·8 kg. An injection of 50 mg. of Pa C (18 mg. per kg.) caused a fall in blood pressure of over 100 mm. of mercury and doubled the respiratory rate. Forty-five minutes after this injection 50 mg. of Pa crude (18 mg. per kg.) were injected and caused the same type, but much less marked changes in blood pressure and respiration.

with Pa crude the result differed from those of Florey *et al.* in that the pressure fell steeply before recovering, and eventually showing a rise above the original level.

Fig. 1, 2, 3, 4 and 5 illustrate the results obtained with Pa A, Pa B, Pa C and Pa crude, from which it would appear that Pa C is largely responsible for the fall of blood pressure associated with the injection of Pa crude.

Effect on isolated smooth muscle.

In these experiments, one horn of the uterus of a guinea-pig was suspended in 50 ml. of oxygenated Ringer's solution in a container immersed in a water bath, the temperature of which was maintained at 37° C. The substances to be tested were dissolved in Ringer's solution and injected through a glass tube, the opening of which was just above the oxygen inlet and below the suspended segment of uterus.

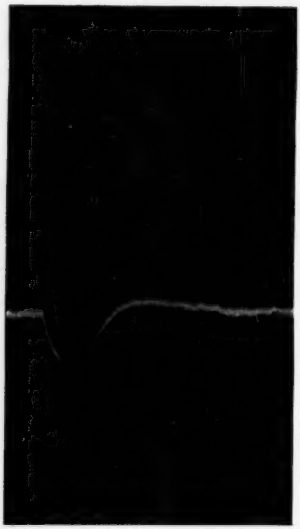

FIG. 5.—*Cat* M/2 (decerebrate). Weight 2·4 kg. An injection of 50 mg. of Pa crude (21 mg. per kg.) caused a pronounced fall in blood pressure, followed by a slight rise above the original level and an increase in respiratory rate and depth.

Fig. 6, 7, 8 and 9 show the type of response obtained in each case. The dilutions given in the tracings are the final dilutions present in the fluid surrounding the muscle. The time marker interval in all figures = 30 seconds.

Effect of Pa crude.

Fig. 6 shows the type of response obtained with a concentration of 1/10,000 of Pa crude.

A concentration of 1 in 25,000 depressed the amplitude of uterine contractions, and a concentration of 1 in 5000 caused them to cease almost entirely. In both cases recovery occurred after washing with warm Ringer's solution.

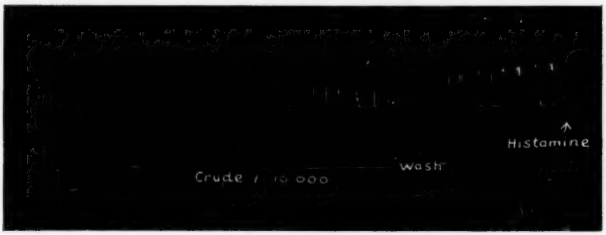

FIG. 6.

Effect of Pa A.

Fig. 7 shows the type of response obtained with Pa A at a concentration of 1 in 10,000. There was a slight decrease of amplitude of the contractions and increase of their frequency. A concentration of 1 in 25,000 had no noticeable effect on the uterine contractions.

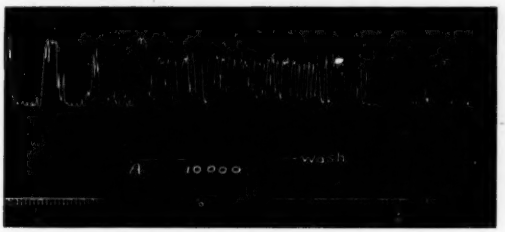

FIG. 7.

Effect of Pa B.

The type of response following contact with Pa B is shown in Fig. 8. A concentration of 1 in 10,000 of Pa B caused a marked contraction of the uterus, which was maintained until the uterus was washed with fresh Ringer's solution, after which the original rhythm returned.

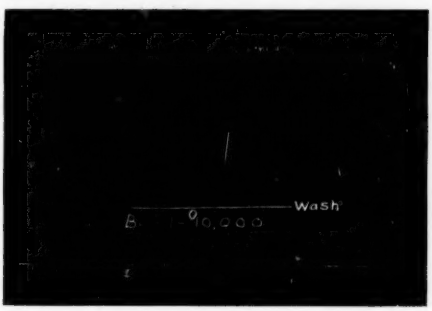

FIG. 8.

A concentration of 1 in 25,000 did not significantly alter the amplitude or frequency of contractions, but when this same muscle was subjected to a concentration of 1 in 25,000 of Pa C the contractions practically ceased.

Effect of Pa C.

Fig. 9 shows the effect of Pa C at a concentration of 1 in 12,500. A concentration of 1 in 100,000 caused an appreciable decrease in the rate of uterine contractions, whereas a concentration of 1 in 50,000 practically abolished the contractions. However, even after being subjected to a concentration of 1 in 12,500 for 20 minutes, rhythmic contractions were restored after washing with Ringer's solution.

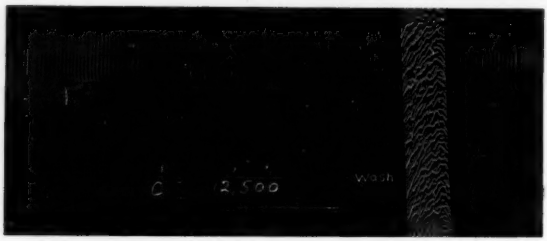

FIG. 9.

It is thus clear that the inhibition of the rhythmical contractions of the guinea-pig uterus by Pa crude can be attributed to its content of Pa C, as both Pa A and Pa B stimulate rather than inhibit contraction.

Excretion in Mice.

Methods.

Two mg. dissolved in 0·5 ml. of saline were administered by stomach-tube to each of three mice. Pa A, Pa B and Pa C were each given in this way. Two mg. of Pa A in 0·5 ml. of saline were also injected subcutaneously into each of three 20 g. mice.

After 5 hours the mice were killed and the urine collected and assayed for antibacterial activity on cylinder-plates seeded with *C. xerosis*. The plates also carried solutions of known strength in terms of which recovery was calculated. The gastro-intestinal tract was removed from mice that had received substance orally and rinsed with 2 ml. of saline. These washings were also assayed.

Results.

The figures given below, which show the amount of substance recovered, are the averages of determinations on each group of three mice. The errors in their estimation are probably large.

Substance.	Route.	Percentage recovered in	
		Urine.	Gastro-intestinal washings.
Pa A . .	Oral .	20 .	3
Pa B . .	,, .	20 .	1½
Pa C . .	,, .	2½ .	3
Pa crude .	Subcutaneous .	20 .	—

Detection in the blood after subcutaneous injection in mice.

The blood was collected from the heart of mice that received Pa A subcutaneously in the previous experiment when the animals were killed. It exhibited no inhibition (1) against *Staph. aureus* when tested by the capillary tube method (Fleming, 1944), or (2) against *C. xerosis* when tested by the cylinder-plate method. The lowest concentrations detectable by these methods were approximately 1 : 40,000 by method (1), and 1 : 160,000 by method (2).

In another experiment each of three 18 g. mice was given 2 mg. of Pa A subcutaneously. One mouse was killed 15 minutes after the injection, and the others 30 and 60 minutes after injection. Samples of heart blood were assayed by methods (1) and (2) above. No definite activity was detected in any of the samples, but there was a small, questionable zone of inhibition on the *C. xerosis* plate around the cylinder of the 1-hour sample.

Protection Tests in Mice.

Owing to the smallness of the supplies available, only one protection test could be carried out with Pa A. For this the pneumococcus was chosen, as it was the most sensitive of the bacteria that had been tested *in vitro*, and a large dose of organisms was not needed to kill mice. Six groups of 10 mice weighing about 20 g. were infected intraperitoneally with 0·2 ml. of a 1 in 10,000 dilution of an 8-hour culture of *Str. pneumoniae* (Wellcome CN 33). Thirty minutes after infection the first dose of Pa A was injected subcutaneously. The injections were repeated every three hours for 48 hours. Table III shows that the largest dose of Pa A given subcutaneously afforded some slight protection, and probably better protection could have been obtained by a larger dose. But the margin between toxic and protective doses is evidently small.

TABLE III.

Single dose, mg.	Route.	Number of mice surviving at the end of hours:								
		18	24	30	36	42	45	57	63	69
0·1 .	Subcutaneous .	10	7	3	2	0	..	..	..	..
0·2 .	,, .	10	10	4	2	1	0	..	..	..
0·4 .	,, .	10	10	9	4	2	0	..	..	..
0·8 .	,, .	10	10	10	10	10	7	2	1	0
0·1 .	Oral .	10	4	0	..	..	..	..	..	..
	(Control) .	10	8	3	1	0	..	..	..	..

SUMMARY.

Proactinomycin A and Proactinomycin C had approximately the same antibacterial activity against the organisms tested, while Proactinomycin B was about half as active as the other two.

The substances were shown to be relatively toxic when injected intravenously into mice, and in sufficient concentrations to decrease the motility of human leucocytes, to depress the blood pressure of decerebrate cats and to alter the normal contractions of the guinea-pig uterus. In all of these tests Proactinomycin C was found to have the most pronounced and Proactinomycin A the least pronounced effect. Similarly Proactinomycin C was the only one of the three substances that decreased phagocytosis of bacteria by leucocytes in the concentrations used. Proactinomycin A caused severe disturbance when injected in sufficient quantity into the cerebro-spinal fluid of rabbits, whereas the same amount of Proactinomycin B or Proactinomycin C had no noticeable effect.

All three substances were detected in the urine and gastro-intestinal washings of mice after oral administration, and Proactinomycin A (the only one tried) was detected in the urine after subcutaneous injection.

In the doses used, Proactinomycin A offered little protection to mice infected with *Str. pneumoniae*. Larger doses would have approached the toxic level.

In view of the low antibacterial power relative to toxicity, it is unlikely that any of the three substances investigated would be useful clinically as antibacterial agents.

We are indebted to Miss Mavis Bond for carrying out the antibacterial titrations under the direction of Sir Percival Hartley, to Mr. J. Kent and Miss S. Long for technical assistance.

The Medical Research Council provided grants towards technical assistance and the purchase of materials.

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THE GENETIC CHARACTER OF O–D CHANGE IN INFLUENZA A.

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In 1943 Burnet and Bull showed that fluids obtained from initial isolations of influenza A virus by the method of amniotic inoculation gave unusual haemagglutination reactions. Fowl cells were not agglutinated or showed a very low titre of incomplete agglutination, but human or guinea-pig cells were agglutinated to a titre in conformity with the infectivity of the fluid. By transfer in the amniotic cavity at limiting dilutions it is possible to maintain the original, O phase, character. One of the 1942 strains, BEI, is still available in this laboratory in the O form. Without these precautions passage by any method in the chick embryo speedily results in the appearance of the D phase, agglutinating fowl cells to the same titre as human or guinea-pig cells.

In all the isolations of influenza A that have been made in Melbourne since 1942 virus was first obtained in the O phase. Workers in other laboratories have for the most part confirmed this finding (Dudgeon, Mellanby, Glover and Andrewes, 1948). A number of isolations have, however, been reported by the use of allantoic inoculation, with recognition of the virus by fowl cell agglutination (Rickard, Thigpen and Crowley, 1944). Nevertheless, all are agreed that allantoic inoculation is far less effective than inoculation of 13-day embryos in the amniotic cavity, and an interpretation of the significance of the results of Rickard *et al.* has already been published (Burnet and Stone, 1945*a*).

There has been no confirmation from other laboratories of maintenance of a strain indefinitely in the O phase by passage at limiting dilutions. This is necessarily a time-consuming and difficult task not likely to be undertaken by anyone not deeply interested in the specific problems. The D phase or an intermediate always appears at each passage and correct dilutions and appropriate timing of harvesting are needed to maintain the O character. In the light of our experience, a strain whose characters can be readily maintained by chick embryo passage is not in the O phase.

The stimulus to publish the work reported in this paper was the appearance of Magill and Sugg's (1948) paper in which it is claimed that the O–D change has no genetic significance, but represents merely a change which can be produced at will by altering the ionic environment of the virus. Briody (1948), in this laboratory, had previously shown that by simple heating, some strains with O type of agglutination reactions could be converted into material agglutinating fowl cells.

It seemed to us desirable that we should describe our own studies of this type on various O and intermediate phase strains of influenza A. It will be shown

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that influenza A virus can exist in a form which maintains its O character despite the application of any of these methods. It can be converted to the D only by the occurrence of an appropriate mutation in the course of multiplication in chick embryo tissues. It is only intermediate forms which can be modified *in vitro* to give O or D reactions.

We would point out that in our original paper O phase is ascribed to many strains which we should now regard as intermediates. In a more recent paper (Anderson and Burnet, 1947) a strain IAN–O was described which was said to be capable of growing in the O-phase in the allantoic cavity. This interpretation will be shown to be wrong. The strain is, in fact, a particularly good example of the intermediate type of virus which allows the various changes described by Magill and Sugg to be demonstrated.

MATERIALS AND METHODS.

Viruses.

BEL.—Influenza A strain isolated during Victorian epidemic, 1942.

O and intermediate (false O) phase virus was obtained by amniotic inoculation of material which had been maintained in the O phase during 26 amniotic passages at limiting dilutions (method of Burnet and Stone, 1945*b*).

D phase virus had received numerous allantoic passages.

IAN.—Influenza A strain isolated in 1946 from a sporadic case of influenza in Melbourne. The virus used was the substrain previously described as in the *O phase*. It was prepared by allantoic inoculation of material which had received 6 allantoic passages since its isolation in the amniotic cavity. Its properties are described by Anderson and Burnet (1947).

Oc.I.—Influenza A strain isolated from an epidemic in Ocean Island, 1948.

O and intermediate (false O and δ) phase virus was obtained during the first or second amniotic passage of the strain.

The following strains were propagated in the allantoic cavity :

WSE.—Egg-adapted substrain of the classical WS (influenza A) strain.

MEL.—Melbourne strain of influenza A.

LEE.—Classical strain of influenza B.

SW.—Shope's strain 15 of swine influenza virus.

NDV.—Newcastle disease virus isolated by Albiston and Gorrie, 1931.

Haemagglutination titrations.—Equal volumes (0·25 ml.) of virus dilutions and 1 per cent red cells were mixed and allowed to settle. Readings were made by observing the pattern of sedimented cells as described by Burnet (1942). Partial (+) agglutination was taken as the end-point. Tests were carried out at room temperature or at 4° C. as specified in the text. Comparative titrations were made with fowl and guinea-pig cells and the titres are expressed as ratio of the two end-points, F/G.

Amniotic inoculation.—The techniques have been described in previous communications from this laboratory. After inoculation the eggs were incubated for 40–42 hours at 35° C. Samples of amniotic fluid were then withdrawn at regular intervals until fluids were obtained which agglutinated human cells ; the amniotic fluids were then harvested. The lungs were removed, ground with alundum, suspended in 1 ml. normal (0·9 per cent) saline and lightly centrifuged. The supernatants were used as lung emulsions.

Receptor-destroying enzyme of V. cholerae (RDE) was prepared by the method of Burnet and Stone (1947).

EXPERIMENTAL.

Character of Variants of a Recently Isolated Strain.

The characteristics of an epidemic of influenza in Ocean Island in October, 1948, will be described and the isolation of the virus reported elsewhere.

A detailed study was made of one strain, isolated in the first passage in the form of amniotic fluid with an F/G titre of $<10/480$ when tested at room temperature. When this fluid was titrated in eggs by amniotic and allantoic methods in parallel the results obtained were as shown in Table I.

TABLE I.—*Results of Passage of Ocean Island O Virus by Amniotic and Allantoic Routes at Different Dilutions.*

Dilution of inoculum.	Amniotic route.		Allantoic route.	
	Number infected.*	Phase.†	Number infected.*	Phase.†
10^{-7}	1/5	0	—	—
10^{-6}	4/4	000	0/6	—
10^{-5}	8/8	000	0/6	—
10^{-4}	5/6	000	1/6	D
10^{-3}	—	—	4/6	δ
10^{-2}	8/8	000 D	5/5	δδδDD
10^{-1}	—	—	6/6	δδ DDD
10^{-0}	—	—	5/6	δδDD

* Number giving haemagglutination of guinea-pig cells and number surviving.
† Phase character of representative fluids (Burnet and Stone, 1945*b*).

It will be seen that the situation is similar to that reported for earlier strains in the O phase.

Eighteen embryo lung emulsions which gave no agglutination of fowl cells at a dilution of 1 : 10 at room temperature were tested at 4° C. Seven of these gave no agglutination at 1 : 10, with agglutinin titres for guinea-pig cells ranging from 100 to 320. Five others gave fowl cell titres at 4° C. which were one-tenth or less of the guinea-pig cell titres. These twelve can be regarded as true O phase virus. Six lung emulsions gave the following values for fowl cell agglutination at 4° C, >80, >80, 80, 40, 40, 20. These last which gave no apparent agglutination at room temperature (18 to 20°) are referred to as " false O " phase virus. As was pointed out in the paper by Burnet and Stone (1945*b*) it is impossible to draw sharp dividing lines in classifying the actual materials, amniotic fluid or embryo lung emulsion, from infected embryos. Preparations can be obtained showing every graduation from " true O " which shows no fowl cell agglutination at any temperature to the full D phase.

In vitro *Characteristics of " True O " Phase Virus in Contrast to " False O " Phase Virus.*

Parallel experiments were carried out on (1) O and other variants of our standard strain BEL, which has been maintained in the O phase for seven years.

(2) The strain IAN–O which might be called the most blatantly false O in our possession and (3) the new strain Ocean Island 1948 (Oc.I.).

Influence of temperature at which haemagglutinin titration is made.

Our primary criterion of true O behaviour has been the absence of agglutination of fowl cells at 4° C. by a 1 : 10 dilution of virus giving a titre of at least 80 with guinea-pig or human cells. When false O strains, e.g. IAN–O, are studied at room temperature, close observation will show that in the early stages of settling a definite pattern is formed on the bottom of the tube. By the time settling is complete there is at most only a slight roughness of the edge of the central " button " of cells. If the cells are reshaken and again allowed to settle there is no trace of agglutination at any stage. This finding, which is common to all the false O viruses tested, provides the clue to the understanding of the behaviour of these strains, viz. that they very rapidly destroy by enzymic action the small proportion of fowl cell receptors to which they can be adsorbed. Anything which prevents or slows down this elution of virus by its own enzymic action will increase the apparent haemagglutinating action on fowl cells.

Apparent conversion of false O to D by treatment of the virus to reduce its enzymic activity.

By appropriate treatment any influenza virus can be converted into an indicator strain, still producing haemagglutination to nearly full titre, but lacking enzymic activity and being inhibited by those mucoid materials which serve as a substrate for the untreated virus (Stone, 1949*a*). Simple heating will accomplish this with some strains, others require to be diluted in a mildly alkaline medium containing citrate or oxalate. When these methods are applied to O and false O materials, the former show no development of a capacity to agglutinate fowl cells. The false O viruses characteristically develop a power to agglutinate fowl cells at room temperature to a titre approaching that to which guinea-pig, human or pigeon cells are agglutinated. The titre is never greater than that given by the untreated virus with fowl cells at 4° C. Results with Oc.I. and IAN are shown in Table II.

TABLE II.—*Effect of Heat on the Agglutination Titres with Fowl and Guinea-pig Cells of True O and False O Virus.*

Strain.	Phase.	Treatment.	F/G titres at room temperature.	
			Original.	After heating.
Oc.I.	O	56° C. 45 min.	<10/210	<10/80
Oc.I.	False O	,,	<10/430	40/160
IAN	,,	52° C. 15 min.	<10/120	140/120

With BEL false O strains, heating to 52° for 15 minutes in the presence of 1 per cent potassium oxalate allowed agglutination of fowl cells at room temperature, but the agglutination was of weaker type than that shown with guinea-pig cells. Similar treatment of the true O phase virus produced no capacity to agglutinate fowl cells.

The method used by Magill and Sugg (1948), suspension of virus in McIlwaine's phosphate-citric acid buffer at pH 5·6 was applied to the Oc.I.–O and false O

material used above (Table II); F/G titres at room temperature produced by this treatment were <10/210 and 80/430 respectively.

Very mild treatment of IAN false O, including incubation for an hour at 37° C. in the presence of borate buffer pH 8·5 plus 0·5 per cent sodium citrate, allowed a full titre D type of agglutination of fowl cells at room temperature.

Agglutination of fowl cells treated with periodate.

Fazekas de St. Groth (1949) has shown that at a certain level of periodate treatment red cells adsorb influenza viruses irreversibly, the normal enzymic action failing to occur. By treating a 25 per cent suspension of fowl cells with an equal volume of M/4000 KIO_4, cells were obtained which at room temperature were agglutinated by false O specimens of IAN or Oc.I to the full titre, and by BEL false O to about half the titre obtained with normal fowl cells at 4° C. True O phase virus of either Oc.I. or BEL was without effect on such cells.

The position in the receptor gradient of O and false O variants.

Previous experience in testing O and D phases of the same strain for their position in the receptor gradient of cells, human or guinea-pig, susceptible to agglutination by both has generally shown that the two phases lie fairly close to one another, the O being earlier than the D (Burnet, McCrea and Stone, 1946). In view of the results described above it was of considerable interest to compare the position in the gradient for fowl cells of false O and D phases of the same strain. Since true O never shows any agglutination of fowl cells it cannot be tested. The experiment with BEL, false O and D, which is shown in Table III followed the usual plan of such tests. Fowl cells were treated with graded amounts of the vibrio filtrate, RDE, and the action stopped by the addition of citrate. After washing with saline the cells were made up to 1 per cent suspensions. Cells were also treated with BEL false O and NDV until stablized. A modified series of viruses was then used to test the agglutinability of each type of cell at 4° C., five agglutinating doses (A.D.'s) being used. The false O material (BEL) was in the form of an embryo lung emulsion with a titre of 1600 with guinea-pig cells. The fowl cell titre at room temperature was <10, at 4° C., 200. It can be seen from Table III that BEL false O is at the " extreme left " of the gradient, quite separated from the D form of the same strain.

TABLE III.—*Relative Positions of BEL false O and BEL–D in Fowl Red Cell Receptor Gradient.*

Red cells treated with.	Haemagglutination by 5 A.D.'s at 4° C.					
	BEL false O.	NDV.	MMF.	WSE.	BEL–D.	SW.	LEE.
RDE							
1 : 10	−	−	−	−	−	−	−
1 : 50	−	−	−	−	−	−	+
1 : 250	−	−	−	−	++	++	++
1 : 1250	−	+	++	++	++	++	++
BEL false O	−	++	++	++	++	++	++
NDV	−	−	+	++	++	++	++
Control	++	++	++	++	++	++	++

DISCUSSION.

The results reported in this paper will, we hope, clarify the confusion in regard to the genetic significance of the O–D series of changes in influenza A viruses. In a subsequent paper a detailed study of the enzymic activity of O, D and intermediate phases of BEL will be reported. This has given results in line with the interpretation which can be placed on the findings given in this paper.

Our view is simply that when first isolated from human material influenza A virus particles are incapable of adsorption to and therefore of enzymic action on the receptor substance of fowl or mouse erythrocytes. In any large population of such virus particles a proportion of mutants appear which are capable of adsorption to the most " accessible " of the fowl cell receptor groupings. This proportion presumably is selectively favoured in the environment of the chick embryo and, in accordance with the now generally accepted views on microbial variation, further variants appear within the favoured clones by which the changed character is accentuated (Demerec, 1945). As the change goes on the virus becomes capable of adsorption to progressively less accessible receptor groupings until it finally reaches its appropriate position in the fowl cell gradient of fully " egg-adapted" strains. In the early stages only a few receptor groupings are accessible and a brief period of contact between virus and cells results in their enzymic destruction with disappearance of the initial cell aggregation. Anything which hinders this enzymic destruction of the most accessible receptors will at this stage of virus adaptation produce an apparent conversion of virus from O to D. The simplest way to inhibit enzymic action is to lower the temperature, but the same result can be achieved by heating virus in the region of 52–56° C. in an appropriate ionic environment, or by modifying the cell receptors with small amounts of periodate.

Even if it were shown that " true O " virus as obtained in primary isolations by the amniotic method, could be induced to give fowl cell agglutination by some physical or chemical treatment, the data provided in this and earlier papers as to the conditions under which fowl cell agglutinating virus appears would still demand a genetic interpretation. The fact that any true O strain will, on passage, give intermediate and D descendants with complete regularity, yet cannot be induced by any manipulation *in vitro* to agglutinate fowl cells, wholly invalidates the view that the O–D phase variations represent merely transformations in agglutinating activity induced by the ionic environment.

SUMMARY.

Influenza A virus strains in true O phase as isolated from the human subject, cannot be manipulated by physicochemical means to produce fowl cell agglutination.

The behaviour of intermediate forms which show such an apparent conversion is explicable in terms of their enzymic activity on cell receptors.

The change from O to D phase on chick embryo passage is due to processes of mutation and selective survival.

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AYFIVIN: AN ANTIBIOTIC FROM *B. LICHENIFORMIS*: PRODUCTION IN POTATO-DEXTROSE MEDIUM.

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MAGARÃO, Arriagada and Thales (1944) reported that cultures of *Myco. tuberculosis* contaminated by a strain of *B. subtilis* underwent lysis, and that products of the contaminating organism were able to modify the course of tuberculosis in guinea-pigs. Cultures of the *B. subtilis* which had been brought to Oxford were found to contain two species of aerobic spore-forming bacilli. One was a strain of *B. subtilis* and the other was a strain of *B. licheniformis*. When tested by the cross-streak procedure on nutrient agar both organisms inhibited the growth of *Staph. aureus*, *C. xerosis* and *Myco. phlei*, but showed little activity against *Bact. coli* or *Ps. pyocyanea*. Antibacterial substances produced by the strain of *B. subtilis* have already been described (Newton, 1949). The present paper is concerned with an antibiotic formed in certain liquid media by the strain of *B. licheniformis*. This organism was originally called A-5 and the antibiotic has been named ayfivin (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949).

EXPERIMENTAL.

The organism.

The strain A-5 showed the usual characteristics of *B. licheniformis* (Gibson, 1944). It was maintained for routine use on slopes of heart-extract agar, which were stored in the refrigerator until required. Fresh subcultures were made every two weeks.

Assay and unit of activity.

Ayfivin was assayed by the cylinder-plate method (Heatley, 1944). A laboratory strain of *Myco. phlei* was at first used as a test organism, but this proved to be easily overgrown by contaminants and to be very sensitive to changes in pH. *C. xerosis* was finally chosen as the most suitable test organism, and the assay was carried out on plates of Lemco-agar seeded with cultures of this organism which had grown for 24 hours at 37° C. The size of the zone of inhibition varied significantly with the pH of the agar, increasing as the pH was reduced from 8 to 6·5.

The unit of ayfivin was based on the activity of a sample of crude ayfivin hydrochloride. This material was arbitrarily stated to contain 5 units per mg. It was stored dry in the refrigerator and appeared to be quite stable. A solution of ayfivin containing 1 unit per ml. produced zones of inhibition 18 to 21 mm. in diameter on plates seeded with *C. xerosis*. For the purposes of assay, solutions of ayfivin were diluted so that they contained from 0·25 to 1 unit per ml. and their activity was compared with that of the standard preparation.

Medium and conditions of culture.

The organism was grown in a potato-dextrose medium prepared in the following manner : King Edward potatoes were peeled, diced and steamed in tap water for one hour. The resulting mixture was filtered through lint, and glucose was added to the filtrate. Two hundred g. of potatoes and 10 g. of glucose were used for each litre of final solution. The initial pH of the medium was close to 7·0.

The medium was distributed in glass culture vessels of the type described by Clayton, Hems, Robinson, Andrews and Hunwicke (1944). Each vessel contained 500 ml. of medium, which formed a layer 2·5 cm. deep ; the use of shallow layers of culture fluid appeared to be essential for good yields of the antibiotic.

After autoclaving for 30 minutes at one atmosphere the medium in each vessel was seeded with about 2 ml. of a 24-hour broth culture of A-5. It was found advantageous to use large inocula, and each vessel received 2 to 3 ml. of a 24-hour shaken flask culture of the organism in heart extract broth.

The seeded vessels were incubated at 37° C. The antibacterial activity of the culture fluid reached a maximum of from 4 to 8 units per ml. after 5 to 6 days The final pH of the medium was about 7·2.

FURTHER OBSERVATIONS.

Crude ayfivin was extracted from the culture fluid obtained by growing A-5 in potato-dextrose broth (Sharp, Arriagada, Newton and Abraham, 1949). The material was found to inhibit the growth of certain gram-positive bacteria in high dilutions and to have a relatively low acute toxicity to mice (Arriagada, Florey, Jennings and Wallmark, 1949). It was therefore thought desirable to produce ayfivin in larger quantities and to make a fuller investigation of its chemical and biological properties. By arrangement with Dr. D. W. Henderson large batches of active culture fluid were supplied by the Ministry of Supply Microbiological Research Department at Porton. Hills, Belton and Blatchley (1949) found that low yields of the antibiotic were obtained in medium prepared from some batches of potatoes, and they devised a synthetic medium which was more suitable than potato-dextrose broth for the production of ayfivin on a large scale.

SUMMARY.

A strain of *B. licheniformis* isolated from a contaminated culture of *Myco. tuberculosis* produces an antibiotic when grown in shallow layers of potato-dextrose broth. The antibiotic has been named ayfivin.

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AYFIVIN: PRODUCTION IN CHEMICALLY DEFINED MEDIA AND COMPARISON WITH LICHENIFORMIN.

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AYFIVIN is an antibiotic produced by a particular strain (A-5) of *Bacillus licheniformis* (Arriagada, Savage, Abraham, Heatley and Sharp, 1949). The present work deals with the development of a chemically defined medium suitable for the production of the antibiotic in bulk. Potato-dextrose medium had given good results when made from King Edward potatoes which had been stored for 6 to 10 months after harvesting (March to July, 1947), but, when this supply was exhausted, new potatoes of the varieties available at the time, or even King Edwards available later, gave only 25 per cent or less of the activity yielded by old King Edwards. The development of a reproducible medium, not subject to seasonal variation, was thus not only of importance for work on the biochemistry of antibiotic formation, but became an urgent practical necessity.

METHODS.

In general, the techniques were those used by Belton, Hills and Powell (1949) in work on a chemically defined medium for the production of licheniformin by a different strain of *B. licheniformis*, with modifications as follows :

Maintenance of cultures.

Spore suspensions were produced by growth on CCY agar (Gladstone and Fildes, 1940) for 4 days at 37° C. The growth was washed off in distilled water to give an opacity of 25 to 50 (Brown's standards). After heating at 60° C. for 90 min. to kill vegetative forms the viable count was 2 to 5 $\times$ 10^9 spores per ml. Such spore suspensions were prepared in quantities required for inoculation during 3 to 6 months and were stored in the refrigerator. Samples of the first suspension produced were also dried and stored *in vacuo* to preserve the strain in a form which had had as few subcultures as possible from the state in which it was originally received from Oxford.

Culture media.

Potato-dextrose.—Batches of 45 l. were made by scrubbing 20 lb. King Edward potatoes, chipping and suspending in double muslin bags in 36 l. tap water in a 15-gallon enamelled iron tank. After standing overnight the tank was steam heated at 90 to 100° C. for 1 hour. The bag of potato residue was removed and 450 g. glucose monohydrate was added to the liquor, together with tap water to restore the volume to 45 l. The pH was about 7 and was not adjusted further. After standing for 30 minutes the fluid was filtered through one layer of lint or two layers of gauze, distributed in 2 l. conical flasks (1 l. per flask) and autoclaved at 7 lb./in.^{2} for 30 minutes.

Chemically defined media.—Ammonium salts and various sources of carbon, as described in the text, were added to a salt mixture containing $H_2PO_4^-$–HPO_4^-, 6 mM ; citrate, 1·7 mM ; Mg^{++}, SO_4^-, 4 mM ; Fe^{+++}, 70μM ; and Mn^{++}, 13 μM. At first citrate was added as sodium salt (0·5 g./l.) and phosphate as K_2HPO_4 (1 g./l.), but later it was found desirable to modify this procedure (p. 434). The pH was adjusted to 7·2–7·4, but usually fell during autoclaving at 12·5–15 lb./in.^{2} for 20 minutes and was within the range 6·6–7·4 (glass electrode) at the time of inoculation.

The chemicals used were not specially purified, but were of laboratory grade, available in quantity for moderate scale production.

Conditions of cultivation.

All cultures for production of antibiotic were incubated at 37° C. in stagnant liquid media, the average depth being 10 mm. Pilot experiments were carried out with 15 ml. quantities in 50 ml. conical flasks. Production was with 1 l. units in covered enamelled steel trays of dimensions about 25 $\times$ 40 cm.

In flasks the most convenient method of inoculation was to add 10^5 spores/ml. to the basal medium in bulk, followed by aseptic distribution. Separate flasks were used for each sample to be assayed, since it was found that disturbance of the pellicle by sampling led to irregular results on continued incubation. On the larger scale, the inoculum was pipetted into each tray in the hot room, followed by aseptic pouring of the culture medium, which had been sterilized separately

in resistance glass flasks. The trays were not subsequently moved before reaping. On this scale, sampling was possible without moving the tray and without appreciable disturbance of the pellicle. A proportionate inoculum of 10^8 spores per tray was unsatisfactory with the synthetic media, owing to the establishment of contaminating moulds during the long lag phase. The lag was reduced and contamination avoided by using an overnight vegetative culture (10 ml./tray) in tryptic beef digest broth (T.M.B.).

Assay of antibiotic activity and its accuracy.

Ayfivin.—The cylinder-plate technique was used as developed by Arriagada *et al.* (1949), except that tryptic beef digest agar (T.M.A.) was used in place of Lemco agar. The test organism was the Oxford strain of *Corynebacterium xerosis*, and standard preparations of the active material were also supplied by the Oxford workers.

All unknowns were assayed at two or three levels differing by twofold steps with at least three cups at each level. In single assays the standard was used at 0·5, 1·0 and 2·0 units/ml., and was treated similarly to the unknowns. In multiple assays an extra level of standard, 0·25 units/ml., was used and more cups were used at each level. Each cup contained 0·15 ml. sample, and steps were taken to compensate for systematic errors arising from the tendency to smaller zones near the point of drainage of the surface inoculum (Heatley, 1944). Bias was also recognized in multiple assays due to the cups not being filled simultaneously. Brownlee, Delves, Dorman, Green, Grenfell, Johnson and Smith (1948) compensated for this effect in streptomycin assay by an appropriate order of filling. In the present case a correction was determined. This was preferred, since, even for 12 assays set up together, it did not exceed $\pm$ 0·4 mm. in the diameter of a zone, with a coefficient of variation of 20 per cent. The standard error of the deviation of each zone from the linear regression of zone diameter on log dilution was 0·5 to 0·9 mm. (based on 25 to 125 degrees of freedom and not constant from day to day), so that the minimum significant difference between the zones for two assays, each the mean of six cups, was 0·6 to 1·1 mm. It is apparent that although the correction for the time of plating may be important, the error of its determination was an unimportant part of the total error and was usually negligible.

Interpretation of data was usually carried out by graphical methods. In those cases where statistical examination was done, it was found that the slope of the regression line was not constant from day to day (6·5 to 8·5 mm. change in diameter for ten-fold change in dilution). Owing to association of greater deviations from regression with the steeper slopes, the accuracy of the determined potencies was relatively constant, the significant difference being 20 to 25 per cent of the larger value for assays based on six cups per sample carried out at the same time with plates of the same batch. For samples which were not compared directly, but only in terms of a standard preparation, due to determination at different times, the significant difference becomes about 33 per cent of the activity of the more potent material.

Licheniformin.—Assays were carried out by dilution in tryptic beef digest broth (T.M.B.) as described by Callow, Glover, Hart and Hills (1947) with *Mycobacterium phlei* as test organism (Mill Hill strain). The method is unsuitable for detection of differences in potency less than two-fold (Belton *et al.*, 1949).

Chemical analysis of culture media.

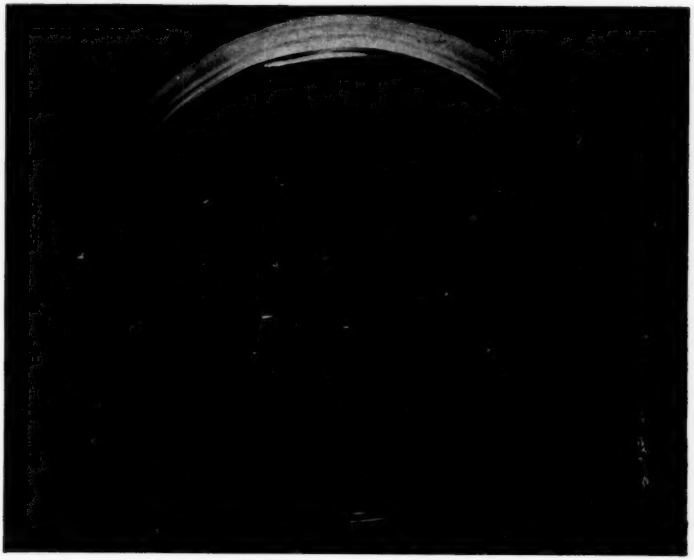
Sugar was determined by the method of Hagedorn and Jansen (1923), after hydrolysis, if necessary, with 0·25 N HCl 10 min. at 100° C., lactate by the method of Friedemann and Graeser (1933) and ammonia using the still designed by Markham (1942).

RESULTS.

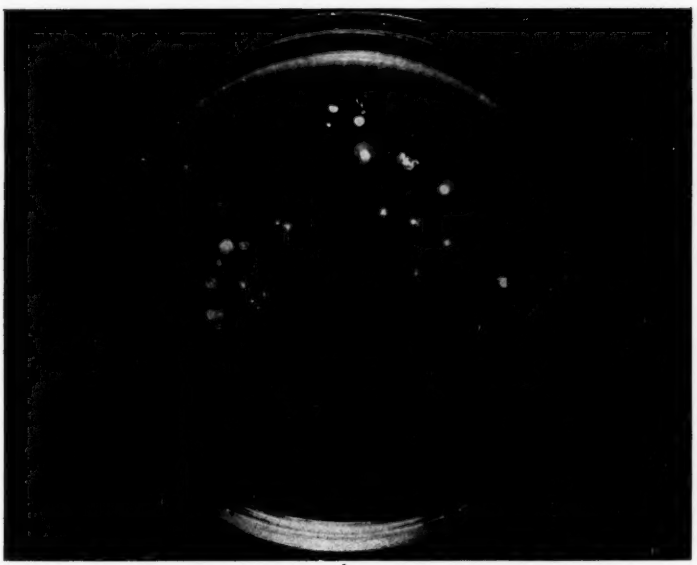
When work was begun on the A-5 strain of *B. licheniformis*, licheniformin (Callow and Hart, 1946 ; Callow, Glover and Hart, 1947 ; Callow, Glover, Hart and Hills, 1947) was known to be produced by another strain (now N.C.T.C. No. 7072). The synthetic medium for licheniformin production (Hart and Hills, 1947 ; Belton *et al.*, 1949) gave less than 1 ayfivin unit/ml. with the A-5 strain, while potato-dextrose then gave 2 to 4 units/ml. The licheniformin-like activity given by the A-5 strain, however, was 700 dilution units/ml. in the synthetic medium and only 90 units/ml. in potato-dextrose. The inverse relationship between the antibacterial activities of the two cultures of the same strain on different media, when compared by different methods, suggested that the principal activities are due to different antibiotics. Since the N.C.T.C. No. 7072 strain behaved similarly to the A-5 strain on both media, it is evident that the production of ayfivin or licheniformin-like materials is controlled largely by the nature of the medium rather than the strain. Although both are identifiable as *B. licheniformis* by the criteria of Gibson (1944), e.g. in failure to liquefy gelatin and capacity for anaerobic growth, the similar behaviour in antibiotic production is unlikely to be due to a common origin ; they are easily distinguishable in colonial form, A-5 producing irregular mucoid colonies, 2 to 5 mm. in diameter on T.M.A. at 22 hours, while on the same medium N.C.T.C. 7072 produces smooth colonies at 16 hours, later giving rise to petal-like outgrowths which coalesce to form a dry radially striated periphery 4 to 7 mm. in diameter (Fig. 1).

Modification of Potato-dextrose.

This medium usually had 22·5 to 26·5 mg./100 ml. total N (16 to 19 mM) and 1·6 per cent total solids, including 1 per cent dextrose. The synthetic medium, on the other hand, had about 60 mM NH_3 and 110 mM total lactate, corresponding to a solid content, including inorganic salts, of 1·65 per cent. The low N content of the former medium accounted for the production of ayfivin, since addition of 100 mM ammonium chloride reduced the peak titre by 50 per cent (Table I), while replacement of the glucose by 100 mM ammonium lactate, to supply the same amounts of C and N as the glucose and ammonium chloride respectively, reduced the titre by 85 per cent. Part of the effect in this case, however, may have been due to the high pH developed in the medium at an early stage, since replacement of the glucose by its C equivalent of sodium lactate led to a 70 per cent. fall in the maximum titre with a rapid fall subsequently, and an even higher final pH. Improvements in the technique of preparing the potato extract led to higher licheniformin titres than were observed in the preliminary experiments described previously, and these tended to be stabilized at a high level, rather than to fall when additional sources of N and C were supplied together. In contrast, ayfivin production tended to show a more definite peak, falling rapidly if alkaline conditions developed with pH exceeding 8, and the highest peak values were observed with a high concentration of C source relative to the N supply.



a



b

Fig. 1.—*Bacillus licheniformis.* 22 hours on tryptic beef digest agar. *a*, Strain A-5; *b*, strain N.C.T.C. No. 7072.

TABLE I.—*The Effect of Additional Sources of C and N on Ayfivin Production in Potato Extract.*

The concentration of all additions was 100 mM, except glucose, 50 mM.

Days.	Additions. None.	NH_4Cl.	Glucose.	Glucose + NH_4Cl.	NH_4 lactate.	Na lactate.
	Ayfivin, units/ml.					
2	5	—	12	10	2	6
4	4	2	22	10	2	7
6	3	2	16	9	3	2
8	2	—	16	8	2	1
	Licheniformin, units/ml.					
4	300	700	1500	800	1000	800
6	450	400	1000	1000	1250	450
8	450	—	500	1250	1250	450
	pH.					
2	8·0	—	6·9	6·9	8·3	8·6
4	9·0	8·3	8·3	7·8	9·0	9·4
6	8·9	8·0	8·5	7·7	8·9	9·7
8	9·0	—	8·7	7·5	8·8	9·7

Modification of the Synthetic Medium.

Proportion of N to C.

Reduction of the proportion of ammonium lactate from 55 per cent to 20 per cent of the total lactate raised the peak ayfivin titre at 4 to 6 days from 2 to 5 units/ml., but the pH was then 9·2 and the titre subsequently fell considerably, as expected from the results with potato media. Replacement of the 100 mM sodium lactate in the modified medium by 50 mM glucose gave a peak titre of 14 units/ml. at 4 to 8 days, falling to 9 and 5 units/ml. at 10 and 12 days respectively, the pH falling from 8·7 to 8·4 during these latter stages of incubation.

It thus appeared that it was possible to reach ayfivin titres in a synthetic medium approaching those previously reached in potato dextrose. Experiments on the optimum concentrations, however, soon showed that even higher titres were possible. The actual concentration appeared to be of less importance than the ratio of C/N, the optimum being in the region of 10 to 15. Various difficulties were encountered in attempting to determine the optima with precision. Thus when the constituents of the medium to be varied were autoclaved separately and mixed aseptically, growth was initiated with difficulty, especially at high glucose concentrations, so that with 120 mM glucose (2·4 per cent) and 108 mM ammonium lactate only 3 out of 5 flasks inoculated showed good growth in 4 days, while with 160 mM glucose and 144 mM ammonium lactate 1 out of 5 flasks showed growth in 4 days, and with 200 mM glucose and 180 mM ammonium lactate only

1 flask showed the first signs of growth at the 11th day and only 3 flasks had visible growth by the 19th day. On the same medium, however, all cultures which grew gave the same titre when tested after the same period from the initiation of visible growth, the optimum being 30 units/ml. or over after 4 to 6 days visible growth with 120–200 mM glucose. This irregular growth was seen with media made up in either tap water or distilled water, and was reduced by adding starch equivalent to 40 mM glucose. It was eliminated completely by autoclaving the tap-water medium after mixing all constituents.

Control of pH.

This was important since little antibiotic was formed before the initial acid fermentation had ceased and the medium had returned nearly to neutrality; on the other hand, a high final pH was to be avoided because of the instability of the active material on incubation in alkaline media. These considerations had given no difficulty during production on potato-dextrose, since the pH at the time of reaping was usually 6·5 to 7·5. In the synthetic medium, however, the pH was usually 8 to 9 before the maximum titre was reached and it was considered that this might be the limiting factor in reaching high titres. Control of pH was

TABLE II.—*The Effect of Buffer Concentration on Ayfivin and Licheniformin Production in Synthetic Media.*

The C and N sources were approximately 120 mM glucose and 110 mM ammonium lactate.

Days	mM K_2HPO_4.		
	6	12	25
	Ayfivin, per cent geometric mean at 4 to 6 days with 6 mM K_2HPO_4.		
2	7	3	0
3	78	55	36
4	107	92	87
5	103	74	82
6	90	66	67
	Licheniformin, units/ml.		
4	1500	800	800
5	1500	600	800
6	1250	800	1000
	pH.		
0	6·9	7·0	7·1
2	6·1	6·2	6·5
3	7·6	7·7	6·9
4	8·6	8·6	8·4
5	8·9	8·9	8·8
6	8·9	9·0	8·9

not easy in media containing such high concentrations of glucose (4 per cent) as were found to be optimal. Increase of buffer concentration to 4-fold merely delayed antibiotic production in certain media by retarding the return to neutrality after the initial development of acidity (Table II). At 3 days the titres were significantly lower both with 12 mM buffer ($P = 0{\cdot}98$) and with 25 mM ($P > 0{\cdot}999$). Although $CaCO_3$ was better in reducing the initial development of acidity, and possibly allowing an earlier reaping, it was still necessary to determine the optimum reaping time with precision to avoid the rapid decline due to the subsequent development of alkalinity.

Attempts were made to control the initial development of acidity by replacing the glucose partly or wholly by less rapidly fermented carbohydrates, starch, glucose and maltose, as with lactose in penicillin production. No significant effect was observed with starch. Table III shows that over the period 4 to 7 days, sucrose

TABLE III.—*The Effect of Different Sugars on Ayfivin Production.*

The ayfivin titres are expressed as per cent of the relatively constant value with sucrose at 4 to 7 days (geometric mean). The values given are the geometric means for 2 experiments except at 7 days, where results from only one experiment were available. The observed differences between the values for the two experiments at 4, 5 and 6 days, excluding the small assays with lactose, showed no significant heterogeneity of variance (Bartlett's test ; $P = 0{\cdot}4$), and a pooled estimate, based on 21 degrees of freedom, was used in estimating the significance of differences.

Sugar.	Concentration, mM.	Ayfivin, as per cent that with sucrose.					Probability of lower geometric mean due to chance.
		4 days.	5 days.	6 days.	7 days.	Geometric mean.	
Glucose	100	46	86	85	60	68	0·01
Sucrose	50	87	97	119	100	100	—
Maltose	50	52	55	33	56	47	<0·001
Lactose	50	1	9	5	32	7	<0·001
Glucose	50	41	48	40	25	39	<0·001
Glucose	50						
+ sucrose	25	56	96	105	93	85	0·15
Glucose	50						
+ maltose	25	66	96	106	79	85	0·15
Glucose	50						
+ lactose	25	25	34	33	25	29	<0·001

was, on the whole, significantly better than its C equivalent of glucose ($P = 0{\cdot}99$), mainly due to the earlier development of high activity ; the apparent earlier decline with glucose was probably not significant ($P = 0{\cdot}07$). Lactose, as would be expected from fermentation tests in the *Bacillus* group, was not an effective source of C. Mixtures of glucose with either sucrose or maltose were not significantly different from either sucrose ($P = 0{\cdot}3$) or glucose alone ($P = 0{\cdot}18$). Subsequently, when media were developed in which a high final pH was either avoided or delayed, sucrose showed no consistent advantage over glucose.

This control of pH made use of the observation that cultures with ammonium chloride as sole source of N became very acid ($pH < 5$) and produced no anti-

biotic. If the amount of N supplied as ammonium lactate was insufficient and the remainder was supplied as ammonium chloride, a suitable balance of these two salts ensured a final pH which did not rise above 7 (Table IV). No improvement in the peak antibiotic titre occurred, however, until the sodium citrate and potassium phosphate of the basal medium were replaced by the acids or their ammonium salts, the adjustment of the initial pH of the medium being made with ammonia rather than NaOH. Na^+ and K^+ were supplied as chlorides.

TABLE IV.—*The Effect of Ammonium Chloride on Antibiotic Production and pH Changes.*

All media had 200 mM glucose.

	Medium A. 1·7 mM Na_3 citrate. 5·0 mM K_2HPO_4.			Medium B. 1·7 mM Am_3 citrate. 5·0 mM NaCl. 5·0 mM Am_2 HPO_4. 10·0 mM KCl.		
Ammonium salts, mM { Chloride	33	33	0	33	33	0
Ammonium salts, mM { Lactate	40	80	80	40	80	80
Days.	Ayfivin, units/ml.					
3	8	5	8	5	13	12
4	8	16	26	9	20	22
5	33	34	34	32	47	47
6	23	21	24	28	33	38
	pH.					
0	6·7	6·1	6·3	6·7	6·7	6·3
3	5·3	6·0	6·0	5·6	5·4	6·4
4	5·5	6·4	7·0	5·7	6·0	7·4
5	4·9	7·4	7·9	5·9	6·1	7·9
6	5·9	8·2	8·5	6·2	6·3	8·3

Optimum concentrations of glucose and ammonium lactate.

These optima were interdependent as shown in Fig. 2 to 4, which incorporate the results of 7 independent experiments. Fig. 2 shows the concentrations, in the presence of ammonium chloride, at which the maximum titres equalled or exceeded 10, 20 and 30 Oxford units/ml. respectively. In order to avoid giving excessive weight to exceptionally high assays, the maximum has been taken as the geometric mean of the two consecutive highest daily titres in the period 4 to 6 days, or the geometric mean of all three where these did not differ significantly. At low concentrations of glucose and ammonium lactate, the maximum was reached in 4 to 5 days, while at high concentrations 5 to 6 days were usually necessary. Fig. 3 and 4 show some of the actual values observed, as opposed to the limits within which they occur, at selected concentrations. With glucose concentrations in the neighbourhood of 125, 200 and 265 mM, the optimum ammonium lactate concentrations were 35, 50 and 80 mM respectively, but the upper limit was not

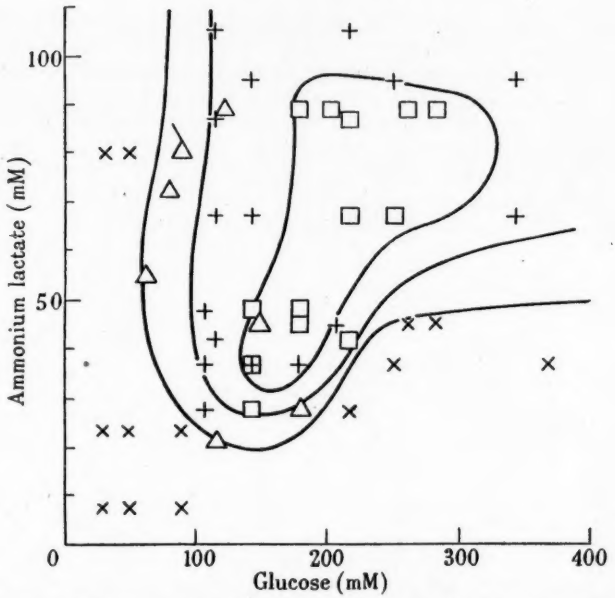


FIG. 2.—The effect of glucose and ammonium lactate concentrations on ayfivin production in the presence of ammonium chloride. Less than 10 units/ml. × ; 10–20 units/ml △ (single obs.) ; △ (two identical observations) ; 20–30 units/ml. + ; over 30 units/ml. □. The curves show the concentrations at which the maximum yields were 10, 20 and 30 units/ml.

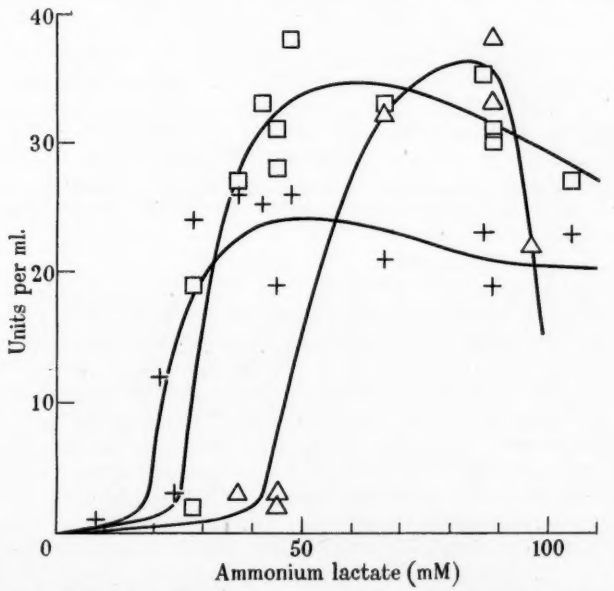


FIG. 3—Optimum ammonium lactate concentrations for ayfivin production at different glucose concentrations in the presence of ammonium chloride. Glucose concentration (mM) : 125 + ; 200 □ ; 265 △

sharply defined, except possibly at the highest glucose concentration (Fig. 3). The optimum glucose concentration was more definite (Fig. 4), and increased only from 190 mM to 260 mM as the ammonium lactate concentration increased from 40 to 80 mM, but the titres were most sharply limited by excess glucose at low lactate concentrations. These critical concentrations extended from the region of 30 mM lactate and 180 mM glucose to 45 mM lactate and 250 mM glucose, so that combinations of lactate and glucose concentrations, lower and higher respectively than this boundary, gave practically no antibiotic due to acid formation (pH 4·5 or less) which was not reversed on further incubation.

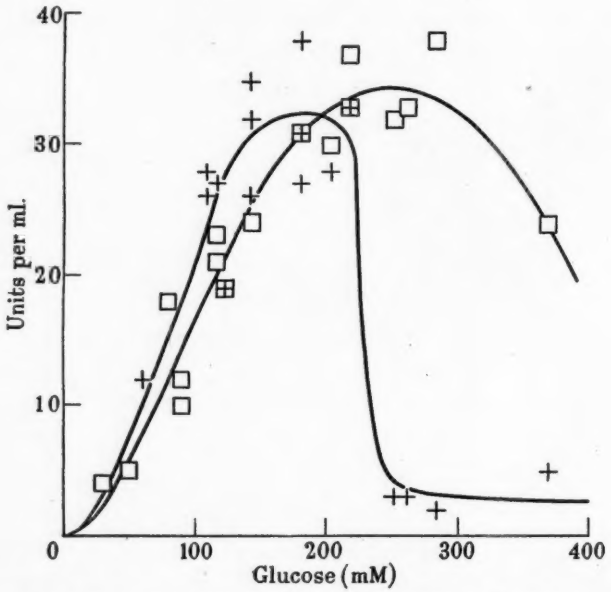


FIG. 4.—Optimum glucose concentration for ayfivin production at different ammonium lactate concentrations in the presence of ammonium chloride. Ammonium lactate concentration (mM): 40 + ; 80 □.

Consumption of Nutrients.

Table V shows the consumption of glucose and ammonia in parallel with ayfivin formation and pH changes at ammonium lactate concentrations 28 to 89 mM with approximately 180 mM glucose (Exp. 1) and with 45 mM ammonium lactate and 163 to 285 mM glucose (Exp. 2). Exp. 1 also shows the changes in lactate concentrations in some cases. Although much of the glucose was consumed before high ayfivin titres were reached, at least 10 μmol/ml. usually remained at the time of maximum antibiotic production. Consumption was delayed at glucose levels above 180 mM and considerably so at 285 mM. Ammonia consumption, on the other hand, was less influenced by its initial concentration or that of glucose, except at high glucose levels (285 mM) in the presence of insufficient ammonium

TABLE V.—*The Consumption of Nutrients during Ayfivin Production.*

All media contained approximately 30 mM ammonium chloride.

Experiment.	Initial concentration (mM). Glucose.	Lactate.	Days.	Ayfivin (units/ml.).	Change in nutrients (μ mol./ml.). Glucose.	Lactate.	NH_3.	pH.
1	180	28	0	—	—	—	—	7·5
			3	3	−101	+17	−34	5·1
			5	4	−162	+19	−34	4·6
	180	36	0	—	—	—	—	7·2
			3	15	−143	+14	−45	5·5
			5	21	−171	−12	−66	5·1
	180	49	0	—	—	—	—	7·4
			3	23	−148	−1	−52	5·8
			5	40	−172	−30	−69	5·3
	180	89	0	—	—	—	—	6·7
			3	8	−113	—	−46	6·5
			5	22	−171	—	−82	8·2
2	163	45	0	—	—	—	—	6·9
			3	4	−104	—	−39	6·0
			5	31	−151	—	−60	5·0
	205	45	0	—	—	—	—	6·8
			3	5	−90	—	−35	6·1
			5	27	−186	—	−59	5·9
	285	45	0	—	—	—	—	6·7
			3	3	−36	—	−23	6·2
			5	3	−156	—	−40	4·7

lactate (45 mM) to allow antibiotic formation. Good antibiotic titres were reached with a consumption of glucose of 150 to 190 μmol/ml. and of ammonia 60 to 80 μmol/ml. At these levels lactate consumption was 10 to 30 μmol/ml., but was replaced by accumulation of lactate where the utilizable ammonia was inadequate in relation to the available supply of glucose. It may be concluded that about 15 atoms of C were required for the utilization of 1 atom of N under conditions where good ayfivin production occurred.

Application to Production.

Before conditions were found for preventing the final alkaline drift of the culture, production of 200 l. batches was begun on a synthetic medium containing 32 g. glucose monohydrate and 22·5 ml. 50 per cent ammonium lactate per l. Analysis of the autoclaved medium gave about 155 mM glucose, 125 mM lactate and 145 mM ammonia. The assay of 10 consecutive batches, ranging from 8·0 to 13·4 units/ml., had a geometric mean of 10·26. Of these batches, 5 reaped at 4 days gave a geometric mean of 10·33 not significantly different ($P \approx 0{\cdot}9$) from the 10·19 for 5 reaped at 5 days. These values were also not significantly different from that observed with potato-dextrose medium, 10·1, in 28 consecutive batches, ranging from 6·5 to 14·7 units/ml. The glucose concentration in the synthetic medium was believed to be suboptimal, but higher concentrations were avoided

because they delayed growth, allowing contaminating moulds to become established —a problem which was serious with a light spore seeding, but which was overcome by the use of a heavy vegetative inoculum. In smaller aliquots (15 ml./50 ml. conical flask) there was a rise in titre after the 4th day to about 23 units/ml. at 5 and 6 days, but in larger cultures with the same average depth of medium the values were falling at 6 days. The reasons for this discrepancy have not been investigated.

Following the use of ammonium chloride to control the final pH of the medium, higher titres were reached on the larger scale approaching those reached in small flasks. The optimal concentrations of ammonium lactate and glucose were found to be similar to those previously observed on a smaller scale and K^+ was found to be essential, but not Na^+. The optimal concentration of KCl was reached at 7 mM (Table VI) ; in Exp. 1 lower yields of antibiotic were observed at 2 mM, both with and without Na^+, but Exp. 2 showed no significant heterogeneity with 5 concentrations from 2 to 16 mM (P = 0·35 for 4th day).

TABLE VI.—*Optimum Potassium for Ayfivin Production.*

Experiment.	NaCl (mM).	Days.	Ayfivin, units/ml. with— 2.	4.	7.	10.	16 mM KCl.
1	0	4	12	—	21	—	21
		5	12	—	21	—	17
		6–7	7	—	22	—	17
	11	4	14	—	26	—	—
		5	18	—	29	—	—
		6–7	10	—	26	—	—
2	7	4	12	16	17	12	16
		5	18	15	15	16	16
		6–7	21	17	20	17	21

The medium finally adopted therefore had the following composition :

Glucose monohydrate	44 g.
Ammonium lactate (5·25 M)	10 ml.
$MgSO_4.7H_2O$	1 g.
Iron ammonium citrate (1·7 per cent)	1 ml.
$MnSO_4.4H_2O$ (0·3 per cent)	1 ml.
Ammonium chloride	0·6 g.
Sodium chloride	0·4 g.
Potassium chloride	0·4 g.
Phosphoric acid (sp. gr. 1·75)	0·3 ml.
Citric acid	0·3 g.

Dissolved in tap water and made up to 1 l.

Ammonia (sp. gr. 0·880) . . . . approx. 1·2 ml. to bring pH to 7·3 to 7·4.

After autoclaving (12·5 to 15 lb./in.² for 20 minutes), the analytical figures were 195–205 mM glucose, 54–57 mM lactate and 75–90 mM ammonia, pH 6·3–7·3. The figures for lactate were reproducible only if the strength of the commercial 50 per cent W/W solution was standardized before use. Analytical control of

the other components was not found to be essential since the concentrations were not critical.

After reaping at 5 days, 5 consecutive batches, ranging from 12 to 32 units/ml., gave a geometric mean of 20·8 significantly greater ($P = 0{\cdot}99$) than either the earlier synthetic medium or potato dextrose. At 4 days, although high titres were sometimes reached, the geometric mean, 12·8 units/ml., was not significantly higher than that with earlier media, owing to the scatter, 6·6 to 35 units/ml., of the observations. Any improvement which occurs on further incubation is relatively small, so that not less than 10 to 20 batches would be necessary to establish it with a high degree of probability.

Differentiation of Ayfivin from Licheniformin.

Although licheniformin titres of 1500 units/ml., as high as any previously observed, were reached in some media designed for ayfivin production (Table II), the ratio of licheniformin units to ayfivin units never exceeded 50, compared with about 1000 for a purified sample of licheniformin supplied by Dr. R. K. Callow. In the medium finally adopted, a ratio 3 was more usual and even lower values were observed. Although the cultures were thus practically free from licheniformin (and even the small activity which was observed against the licheniformin test organism may not have been due to the same active principle), it was desirable to establish the differentiation on a firmer basis.

Table VII shows the relative effects of Seitz-filtration and autoclaving, both at pH 2·5, on the activities of culture fluids for licheniformin and ayfivin production, and of purified preparations. The samples were assayed by the cylinder-plate technique against *C. xerosis*. Licheniformin showed a variable loss on

TABLE VII.—*The Effect of Seitz-filtration and Autoclaving on Activity of Ayfivin and Licheniformin-like Preparations.*

Filtration and autoclaving were carried out at pH 2·5 and the samples were readjusted to pH 7 before testing. In filtration, 10 ml. were passed through a Ford Sterimat SB 3·6 cm. in diam. Autoclaving was at 10 lb./in.^{2} for 12·5 min.

Assays were carried out by the cylinder plate technique with *C. xerosis* as test organism.

Antibiotic.	Type of preparation.	Per cent recovery. Filtered.	Autoclaved.
Ayfivin	Culture fluid	86	49
	Partially purified*	87	58
Licheniformin	Culture fluid, A-5 strain, 875 units/ml.	59	229
	Fluid from autoclaved culture, A-5 strain, 2000 units/ml.	91	—
	Partially purified	95†	104†
		—	90‡

* From Dr. E. P. Abraham, School of Pathology, Oxford, and used at 0·5 mg./ml. = 11 Oxford units/ml.

† From Dr. R. K. Callow, National Institute for Medical Research, using strain N.C.T.C. 7072. The concentration was 1·0 mg./ml. = 5000 dilution units/ml.

‡ From Mr. L. H. Kent and Dr. B. T. Tozer of this department, using strain A-5. The concentration was 0·2 mg./ml. = 1000 dilution units/ml.

filtration. The autoclaved culture fluid and purified preparations, which had been isolated from fluid which had been boiled, both showed no significant loss. Unheated culture fluid, however, showed about 40 per cent loss. At present it is not known whether this discrepancy is due to a change in the nature of the material, or whether it is merely due to the increased amount which appears to be produced in the culture fluid as a result of heating. Apart from this increase, changes on autoclaving were insignificant. With ayfivin, on the other hand, there was about 15 per cent loss on filtration under the conditions used and about 50 per cent loss on autoclaving.

The two antibiotics also differ in their diffusibility in agar and in their antibacterial activity against various test organisms. Thus, although good culture fluids for the preparation of either antibiotic inhibit *C. xerosis* in broth at a dilution of about 1/2000, fluids for ayfivin production must be diluted 20- to 40-fold to give the same size of zone as 1 unit/ml. Oxford standard in the cylinder-plate assay, while licheniformin culture fluids give the same size of zone without dilution. These additional dilution factors should be borne in mind in considering the inhibitory dilutions against various test organisms of preparations standardized by the cylinder-plate method (Table VIII). The correspondence between those values given for *C. xerosis* in broth and on the surface of agar shows that the differences between the values for ayfivin and licheniformin for this organism are due to different rates of diffusion, and not to a different type of action against organisms in broth as compared with those grown on the surface of agar. There is good agreement, within the limitations of the methods, between the values

TABLE VIII.—*Antibacterial Activity against* C. xerosis *and strains of* Myco. phlei *of Ayfivin and Licheniformin-like Materials Standardized by Diffusion Method.*

All materials were sterilized at pH 2·5 either by Seitz-filtration or by autoclaving 12 min. at 10 lb./in.^{2} and were adjusted aseptically to pH 6·5–7·5 before testing. The preparations were the same as those of Table VII.

The diffusion unit is the amount of material per ml. of a solution which gives the same size of zone, in the cylinder plate assay with *C. xerosis*, as 1 unit/ml. of an Oxford standard of ayfivin.

The dilution unit is the amount which in 1 ml. culture medium just inhibits a chosen test organism. Culture media were tryptic beef digest agar (T.M.A.) and tryptic beef digest broth (T.M.B.).

The range of the ratio dilution units/diffusion units is given for at least four independent observations.

Antibiotic.	Ratio, dilution units/diffusion units.				
	Corynebacterium xerosis.		*Mycobacterium phlei.*		
	T.M.A.	T.M.B.	N.C.T.C. No. 1471. T.M.B.	Mill Hill. T.M.B.	N.C.T.C. No. 525. T.M.B.
Ayfivin:					
Culture fluid	—	90–120	30–110	4–40	2–15
Partially purified	40–45	40–160	20–80	1–15	0–1
Licheniformin:					
Culture fluid, A-5 strain	—	400–1200	800–2000	200–1400	800–1600
Partially purified, A-5 strain	600–2000	800–2000	2500–4000	450–900	1600–2400
Partially purified, N.C.T.C. No 7072	900–1100	600–3000	500–5000	350–1200	1000–7500

for culture fluids and the corresponding purified materials, showing little qualitative change as a result of isolation. The licheniformin from strain A-5 is similar to that from N.C.T.C. 7072, both inhibiting the strains of *Myco. phlei* used, at dilutions little different from those inhibiting *C. xerosis*. With ayfivin, on the other hand, the Mill Hill strain is relatively resistant and N.C.T.C. 525 is quite resistant, except to culture fluids which may contain traces of licheniformin.

DISCUSSION.

The main finding of this work is that the antibiotic activity of culture fluids of *Bacillus licheniformis* is altered qualitatively as well as quantitatively by the nature of the culture medium. Although other species of organism are known to produce more than one antibiotic, little is known of the part played by the culture medium except in the case of the penicillin family, where the presence of derivatives of phenyl acetic acid is known to favour, under suitable conditions, the production of penicillins with an aromatic sidechain (Higuchi, Jarvis, Peterson and Johnson, 1946). Preparations from different media are also stated to show differences with both tyrothricin (Stokes and Woodward, 1943) and subtilin (Lewis, Humphreys, Thompson, Dimick, Benedict, Langlykke and Lightbody, 1947), but the constituents responsible have not been defined. With *Bacillus licheniformis* it has now been shown that definite changes in synthetic media give rise to culture fluids and concentrates with different antibiotic properties, but it is not yet possible to account for these differences in terms of the chemical structures of the antibiotics concerned. The effect of the medium, however, is not likely to be related to the presence of precursors of specific groups, as with the penicillin family, since the changes may be produced simply by the use of glucose instead of lactate as main source of C with a reduction in the ratio of utilizable N/C from 1/6 to 1/15, the N being supplied as ammonia in both cases. On the medium with lactate and a high N/C ratio, which had previously been designed for the production of licheniformin by strain N.C.T.C. No. 7072 (Hart and Hills, 1947 ; Belton, Hills and Powell, 1949), licheniformin-like activity was also produced by the A-5 strain, and an active hydrochloride was isolated (Kent and Tozer, unpublished) by the method of Callow *et al.* (1947).

On the medium with glucose and a low N/C ratio, the active principle produced by both strains was less stable to autoclaving at pH 2·5 and less active against two more or less resistant strains of *Myco. phlei* (N.C.T.C. 525 and Mill Hill), but was relatively more active when assayed by a diffusion method against *C. xerosis*, presumably owing to greater diffusibility in agar. Concentrates of material from the A-5 strain were characterized as ayfivin (Sharp, Arriagada, Newton and Abraham, 1949).

It is possible that the effect of the C source was related to the pH changes associated with its metabolism rather than the direct effect of a particular chemical structure. Control of pH was certainly important in ayfivin production, but owing to the high concentration of glucose required, the use of buffers or chalk was not entirely satisfactory and periodic aseptic adjustment was impracticable with stagnant, pellicle-forming cultures. The principle finally adopted was the use of balanced concentrations of acidic and basic metabolites. This has already been applied, in a more elaborate form, to the development and maintenance successively of optimum pH values, first for growth of *Penicillium chrysogenum*, and subsequently for penicillin production by the mycelium (Jarvis and Johnson,

1947). Since success in the use of ammonia as source of N depended on choice of concentration and control of pH, reported failures in the cases of other antibiotics may have been due to such causes. This is certainly the case with subtilin, which has now been produced with ammonia as sole source of N (Feeney, Garibaldi and Humphreys, 1948), in spite of earlier reports to the contrary (Jansen and Hirschmann, 1944). With tyrothricin (Lewis, Dimick and Feustel, 1945), although the medium was improved by supplementing N of ammonium sulphate with a smaller proportion from tryptone, good titres were only reached on this type of medium when citrate or malate were also added, although citrate was strongly inhibitory with tryptone as sole source of N. The metabolism of these anions would counteract the acidity arising from utilization of ammonia.

In view of the importance of comparatively small modifications, such as changes in concentration or rigid control of pH, in changing not only the amount but even the nature of the antibiotic produced by *Bacillus licheniformis*, much of the investigation of culture collections for antibiotic organisms may be of limited value due to the use of unsuitable media. Experience with *B. licheniformis* suggests that ordinary laboratory media designed for optimal growth tend either to produce little antibiotic or to do so transiently, the optimum time for harvest being limited and variable. Better results have been attained in media in which growth is initiated with difficulty, as with the A-5 strain at high glucose concentrations, and in which the nutrients are minimal for satisfactory growth. Consequently we are inclined to favour the view that antibiotics may be either by-products of metabolism or products of incomplete metabolism which do not accumulate under conditions most favourable for the producing organism. An analogy is furnished by diphtheria toxin production which is optimal at Fe concentrations suboptimal for growth (Pappenheimer, 1947). The detailed mechanism by which the medium influences the nature of antibiotic formation has not so far been studied. Apart from a direct chemical effect on metabolism, the possibility arises of causing mutation or of favouring the selection of particular variants which may pre-exist in the inoculum. Investigation of this problem is being continued by other workers in this department.

SUMMARY.

A synthetic medium has been devised to replace potato-dextrose for production of the antibiotic, ayfivin, by a particular strain of *Bacillus licheniformis*. The best synthetic medium produced 12 to 32 units/ml. in 5 days at 37° C., compared with 2 to 15 units/ml. in 4 to 6 days on the potato-dextrose medium, subject to variation due to season and variety.

The conditions for ayfivin production were a high C/N ratio of 15 and maintenance of pH in the region of neutrality. These conditions were satisfied in stagnant culture in a medium containing inorganic salts, 0·2 M glucose, 0·05 M lactate and 0·08 M ammonia.

The inorganic requirements were not studied in detail, but K^+ was shown to be essential at a concentration of not less than 2 mM (80 p.p.m.) and Fe^{+++}, Mg^{++} and Mn^{++} were always added.

On a similar medium with a lower C/N ratio of 6, and C supplied as organic anions, which were metabolized to give a high pH, a different antibiotic was produced, with licheniformin-like characteristics shown by lower diffusibility in agar, greater stability and greater potency against some strains of *Mycobacterium phlei*.

We wish to thank Sir Howard Florey for introducing this problem to us, and Dr. N. G. Heatley for help in standardizing the method of assay. Dr. E. P. Abraham and Dr. D. W. Henderson have contributed valued criticism. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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AYFIVIN: EXTRACTION, PURIFICATION, AND CHEMICAL PROPERTIES.

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THE production of ayfivin by the strain A–5 of *B. licheniformis* has been described by Arriagada, Savage, Abraham, Heatley and Sharp (1949), who grew the organism in potato-dextrose broth, and by Hills, Belton and Blatchley (1949), who later devised a more convenient synthetic medium. A method by which preparations of ayfivin with high antibacterial activity have been obtained from the culture fluids of the *B. licheniformis* is given in this paper. The chemical properties of the preparations indicate that ayfivin is a polypeptide.

EXPERIMENTAL.

Extraction.

Preliminary experiments showed that ayfivin was not extracted from the culture fluid by common organic solvents other than butanol or phenol. Adsorption on to charcoal was therefore used as the first step in its isolation. Elution was accomplished by the use of a two-phase mixture of butanol and dilute hydrochloric acid. The ayfivin in the eluate was transferred from water to butanol, and back to water, by appropriate adjustment of the pH of the solutions, and was then precipitated as a picrate.

In the course of these experiments the potato-dextrose medium was replaced by the synthetic medium of Hills *et al.* (1949). No significant difference was noticed in the behaviour of the active material from the two types of culture fluid during the course of purification.

Adsorption on to charcoal.

The culture fluid was clarified in a Sharples centrifuge and the resulting fluid was stirred for 10 minutes with about 0·75 per cent (w/v) of active charcoal (Farnell Grade 14 neutral or Grade LS neutral). The charcoal was then separated in a Sharples centrifuge or on a large filter. Different charcoals varied greatly in their suitability; with certain acid charcoals no active material could subsequently be eluted. The exact amount of charcoal used depended on the culture fluid. The best results were obtained when the amount was sufficient to adsorb from 90 to 95 per cent of the activity. If larger quantities were used, elution became more difficult and the final yield was reduced, presumably because a larger proportion of the antibiotic was then held on the most strongly adsorbing centres.

Elution.

Ayfivin was eluted by stirring the charcoal corresponding to each 100 l. of culture fluid with a mixture of 1 l. of *n*-butanol and 4 l. of 0·2 N aqueous hydrochloric acid. Butanol was adsorbed preferentially by the charcoal from the two-phase mixture. After filtration the eluate consisted almost entirely of water saturated with butanol. The yield of ayfivin in the eluate was from 40 to 50 per cent of that present in the culture fluid.

The partition coefficient [ayfivin in 0·2 N HCl]/[ayfivin in *n*-butanol] is about 10. In consequence, ayfivin eluted by the butanol is largely displaced into the aqueous phase. Here it is excluded from direct contact with the charcoal since the latter is covered by a layer of butanol. The use of the two liquid phases thus greatly favours elution.

A method of eluting substances from charcoal with a mixture of water and an organic solvent immiscible with water, such as benzene, was described by Steenberg (1944). The procedure differed from that used with ayfivin in that the substance to be desorbed was insoluble in the organic solvent by which it was displaced from the charcoal. Ayfivin, which is insoluble in ether and in benzene, was only eluted from charcoal in very poor yield by a mixture of water and benzene, or water and ether.

Little ayfivin was eluted by a solution of 0·2 N HCl saturated with butanol, or by butanol saturated with aqueous 0·2 N HCl. Acid ethanol was at one time used, but loss of activity occurred during concentration of the relatively large volume of solvent required for elution.

Transfer between water and butanol.

The eluate was adjusted to pH 7, saturated with sodium chloride, and shaken with one-fifth of its volume of butanol, which extracted the ayfivin. One fifth of its volume of water and two volumes of ether were mixed with the extract, and the pH of the aqueous phase was adjusted to 2 with hydrochloric acid. The aqueous phase, which then contained the ayfivin, was freed from dissolved butanol by extraction with ether, and its pH adjusted to 5.

Precipitation of crude ayfivin picrate.

The aqueous solution was cooled in ice, and a saturated aqueous solution of picric acid was added until there was no further precipitate of ayfivin picrate. The picrate was filtered and dried *in vacuo*.

Crude ayfivin hydrochloride.

The picrate was decomposed by dissolving it in 0·2 N ethanolic hydrochloric acid. Ayfivin hydrochloride formed a white flocculent precipitate on adding dry ether to the solution. It contained 20 to 30 units/mg. The unit of ayfivin is described by Arriagada, Savage, Abraham, Heatley and Sharp (1949).

Further Purification. Counter-current Distribution Between Solvents.

A number of methods were investigated for the further purification of crude ayfivin. They included precipitation from aqueous solution by salts, chromatography under various conditions, ionophoresis in silica gel (Consden, Gordon and Martin, 1946), and counter-current distribution between solvents. The method

of counter-current distribution between solvents, developed in recent years by Craig and his colleagues (Craig, 1944), was the most promising. Some preliminary results with this method are described here.

Ayfivin appeared to have an isoelectric point at about pH 7·1, and measurements of antibacterial activity showed that its partition coefficient between water and butanol varied rapidly in this region (see below). It was therefore thought possible that the components of crude ayfivin might be separated most efficiently in a system with a pH near to 7. Subsequent experiments showed a striking difference between the amount of resolution obtained in neutral and in acid solution.

Distribution between an amyl alcohol-n-*butanol mixture and water or phosphate buffer at pH* 7.

The amounts of activity found in the two phases when crude ayfivin (from 0·5 mg./ml. to 10 mg./ml.) was distributed between water or $\frac{m}{10}$ phosphate buffer, at pH 7, and a mixture of amyl alcohol and *n*-butanol (4 : 1) indicated that the overall partition coefficient of the active material was close to unity. The activity of crude ayfivin could be raised by eight-tube counter-current distributions in this system. Different batches of ayfivin contained varying amounts of material of low specific activity which congregated at both ends of the series. In general, the most active fractions (of about 30 to 40 units/mg.) were found in Tubes 3, 4, and 5. The accuracy of the method of assay was too low to make an exact analysis of the results on the basis of activity. Nevertheless, comparison of the experimental distribution curve for the activity with theoretical curves for a single substance indicated that the material contained significant amounts of three active substances. A major active constituent with a partition coefficient close to 1 showed a maximum near the centre of the series, while minor constituents appeared nearer the ends of the series. Different batches appeared to contain somewhat different amounts of the minor active constituents. The results of experiments with two batches are described below.

(1) Using separating funnels to hold the solvents, several grammes of crude ayfivin could be purified in one experiment. For preparative purposes the material in each funnel was transferred to the water phase at the end of the distribution, by adjusting the pH to 2 with hydrochloric acid and adding three volumes of ether. The pH of the aqueous solutions was then brought to 5 with sodium hydroxide, and the material from the different funnels precipitated in the form of the relatively insoluble picrate.

Fig. 1 illustrates the distribution of activity and of total material when one batch of ayfivin (Batch A) was subjected to eight transfers in a system composed of equal volumes of mutually saturated amyl alcohol—*n*-butanol (4 : 1) and water. The distribution was begun with 2 g. of ayfivin (22 units/mg.) and the aqueous phase (50 ml.) adjusted to pH 7 ; the alcohol phase was mobile. No change in pH was detected during the distribution. The material appeared to contain three active constituents, which contributed about 15 per cent, 80 per cent and 5 per cent, to the total activity and concentrated in the tubes near the beginning, the centre and the end of the series respectively. The specific activity of the major constituent in Tubes 3, 4, and 5 was about 40 units/mg. Calculations of partition

coefficients from the relative amounts of total material in adjacent funnels (Williamson and Craig, 1947) did not indicate that any of the material was homogeneous.

(2) The results of an eight-tube distribution on a smaller scale and with a different sample of ayfivin (Batch B) are shown in Fig. 2. The crude material, which had an activity of 20 units/mg., was part of a large batch that has been reserved for more extensive distributions.

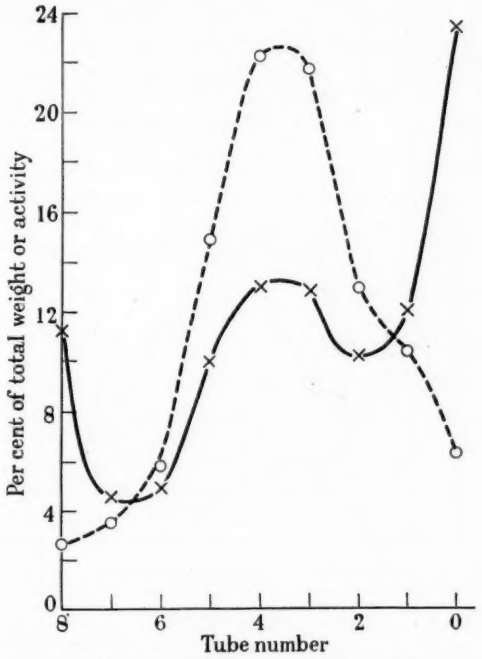

FIG. 1.—Eight-tube counter-current distribution of crude ayfivin (Batch A) between amyl alcohol-*n*-butanol (4 : 1) and water (pH 7). Upper phase mobile.

–×————×– Dry weight.
-○ - - - - - - - - ○- Activity.

The experiment was carried out in stoppered test-tubes. The two phases, each of 8-ml., were amyl alcohol-*n*-butanol (4 : 1) and M/10 phosphate buffer at pH 6·8. The crude ayfivin hydrochloride (100 mg.) was dissolved in a mixture containing rather more than 8 ml. of each phase, and the pH was adjusted to 7 with N NaOH. The volume of each phase was made up to 9 ml., and a small amount of insoluble material was removed by centrifuging. Tube 0 was then filled with 8 ml. of each clear layer, and the remaining 2 ml. of the mixture was used for control determinations of dry weight and activity. The phosphate phase was mobile. At the end of the distribution 0·1 ml. amounts of both phases were removed from each tube and added to water (2 to 4 ml.) for determinations of

activity. The alcohols were extracted from the solutions by *n*-hexane before assay. The remaining alcohol phases were then removed, and the aqueous layers were extracted first with 3/5 volumes and then with 2/5 volumes of *n*-butanol. Each extract was mixed with the original alcohol phase from the corresponding tube. The weight of material in the extracts from each tube was determined by evaporating 4 ml. quantities of the solutions at 100° C. in a stream of air. The weight of phosphate extracted (< 0·05 mg./ml.) was negligible compared with the weight of peptide.

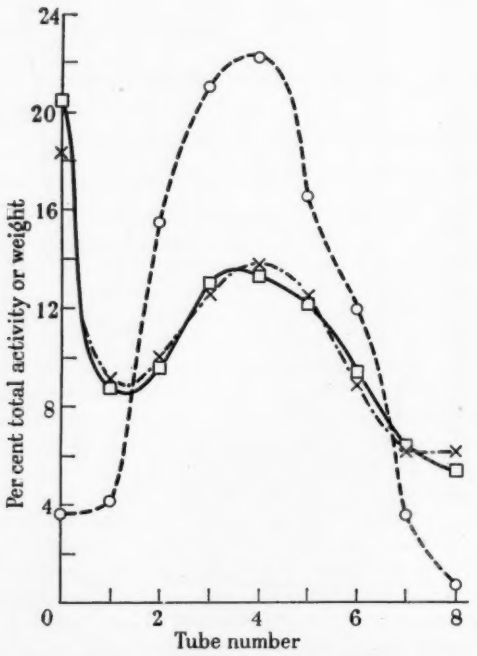

FIG. 2.—Eight-tube counter-current distribution of crude ayfivin (Batch B) between amyl alcohol-*n*-butanol (4 : 1) and m/10 phosphate buffer (pH 6·8). Lower phase mobile.

□———□ Dry weight. ○ - - - - - ○ Activity.
×—·—·—× Dry weight estimated by ninhydrin reaction.

The amounts of material in the different tubes were also estimated by using the ninhydrin reaction in the manner described by Moore and Stein (1948). Aliquots corresponding to from 20 to 150 μg. of material were evaporated to dryness, and the residues hydrolyzed with 0·3 ml. amounts of 20 per cent hydrochloric acid in sealed tubes placed in an oven at 110° C. for 18 hours. After removal of the hydrochloric acid the ninhydrin colours given by the amino acid mixtures were measured in a photoelectric colorimeter, and compared with those obtained from standard amounts of ayfivin. The method has proved convenient for the estimation of small amounts of material, and has given curves rather

close to those obtained by weighing. It will not be reliable if various peptides in a mixture give ninhydrin colours after hydrolysis which differ significantly in intensity.

In this distribution the material with the highest specific activity (about 30 units/mg.) was found in Tubes 3 and 4. The calculated partition coefficients of the material in the different tubes varied continuously, and no tube appeared to contain a homogeneous substance.

Distribution between sec. *butanol and* 0·5 N *acetic acid.*

The system already described had practical disadvantages. Its pH was that at which ayfivin showed a minimum solubility, and several manipulations were necessary to isolate the material in the different fractions, or to obtain it in a form suitable for the determination of dry weight. An alternative system was therefore investigated in which the partition coefficient of the activity had a convenient value. The two phases were prepared by mutually saturating equal volumes of *sec.* butyl alcohol and 0·5 N acetic acid. The pH of the aqueous phase was about 2·8. The system resembles that used by Barry, Gregory and Craig (1948) for the purification of commercial bacitracin.

The partition coefficient of ayfivin in this system expressed as $\frac{\text{activity per ml. in } \textit{sec.} \text{ butanol}}{\text{activity per ml. in 0·5 N acetic acid}}$ was about 0·5. Fig. 3 shows the results obtained from an eight-tube distribution in which the volume of the butanol (4 ml.) was made twice that of the acetic acid (2 ml.) in order to bring the maximum activity near the centre of the series. The material used (80 mg.) was part of Batch B, whose distribution curve in the system at pH 7 is shown in Fig. 2. At the end of the experiment the amount of material in each tube was determined by evaporation of aliquots from the two phases. Activity was measured by the cylinder-plate method after adding 0·1 ml. of the alcohol layer, and 0·05 ml. of the lower layer, to an appropriate volume of water, and extracting the resulting aqueous solution with ether. An alternative method, in which all the material was transferred to the aqueous phase by the addition of 12 ml. of ether to each tube, gave almost identical results.

Fig. 3 shows that the distribution in this system, in contrast to the distribution in the system at pH 7, did not differ greatly from that to be expected if the preparation of ayfivin had consisted of a single substance. The fact that the curve rose somewhat more sharply to a maximum than the theoretical curve for homogeneous material could be attributed to small changes in the relative volumes of the phases, which occurred during the transfers. Except for Tube 8, which contained a small amount of inactive material, the activity-curve was roughly superimposable on the weight-curve. The specific activity of the material isolated from Tubes 3, 4 and 5 was no greater than that of the starting material. Very little resolution had therefore occurred under these conditions.

Further observations.

Although it has been established that the specific activity of crude ayfivin may be raised considerably by eight-tube counter-current distributions at pH 7, the units of activity in the separate fractions have usually appeared to total not

more than 80 per cent of those in the original material. It is thought probable that the loss of activity is partly caused by small amounts of impurities in the solvents used. Inactivation caused in this way becomes more serious the purer the ayfivin and the more dilute the solutions, and has complicated work on the further purification of the material.

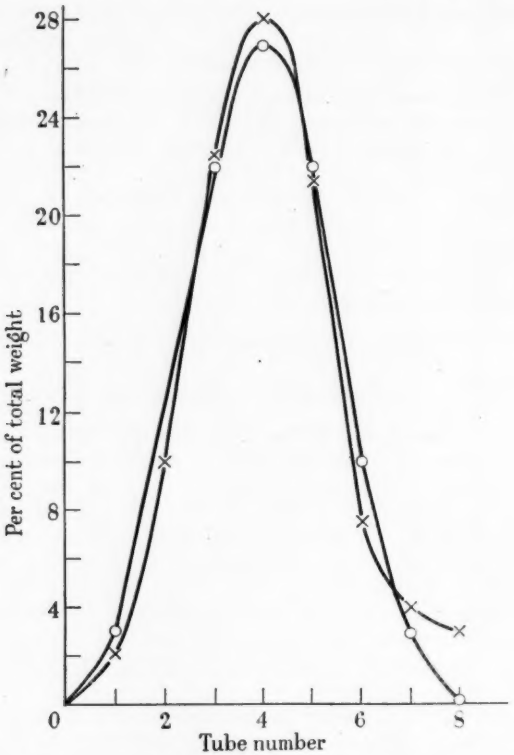

FIG. 3.—Eight-tube counter-current distribution of crude ayfivin (Batch B) between *sec* butyl alcohol and 0·5 N acetic acid. Lower phase mobile.

×————× Dry weight.
○————○ Theoretical curve for a single substance.

Physical and Chemical Properties.

The physical and chemical properties described here refer to preparations of ayfivin with an activity of about 40 units/mg., which were obtained from Fractions 3, 4, and 5 of eight-tube counter-current distributions at pH 7. These fractions contain the main active constituent of the crude material, but there is evidence that they are not homogeneous. Some of the properties will therefore need re-investigation if pure ayfivin becomes available.

Ayfivin hydrochloride gave the following results on elementary analysis:

C, 48·8; H, 7·3; N, 14·6; S, 1·5; Cl, 7·9. It contained 2·2 per cent of amino nitrogen according to the van Slyke method of determination. It showed no strong absorption bands in ultraviolet light, but had a weak blue fluorescence. It was very soluble in water or ethanol, sparingly soluble in acetone, and insoluble in ether. It dialyzed readily through a cellophane membrane.

Free ayfivin was much less soluble in water than the hydrochloride.

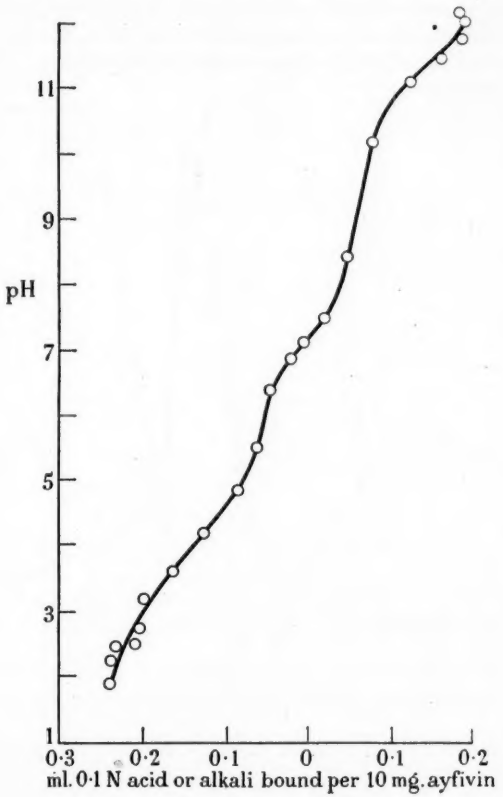

FIG. 4.—Titration curve of ayfivin.

Ionizable groups

Ayfivin behaved as a weak base. An aqueous solution of its hydrochloride had a pH of about 2·5. Electrometric titration, using a hydrogen electrode, showed that it contained groups ionizing between pH 2 and 5, pH 5·5 and 8·5, and pH 10 and 12 (Fig. 4). Changes in the partition coefficient of the activity between water and *n*-butanol when the pH was varied from 5·5 to 8·5 (see below) showed that ionization in this region was a property of the active material itself. The titration curve was compatible with the assumption that ayfivin consisted of

polypeptide. Buffering in the pH range 2–5 could be attributed to the free carboxyl groups of dicarboxylic amino acids; buffering in the pH range 5·5 to 8·5 could be due to the iminazole group of histidine and an α-amino group; and buffering in the pH range 10 to 12 could be due to free amino groups of diamino monocarboxylic acids. The "isoelectric point" of the material was at pH 7·1.

Solubility.

Ayfivin was extracted almost completely from aqueous solution between pH 2 and pH 10 by shaking with an equal volume of phenol saturated with water. It was not extracted significantly from water at any pH by ether, chloroform, amyl acetate, or benzene.

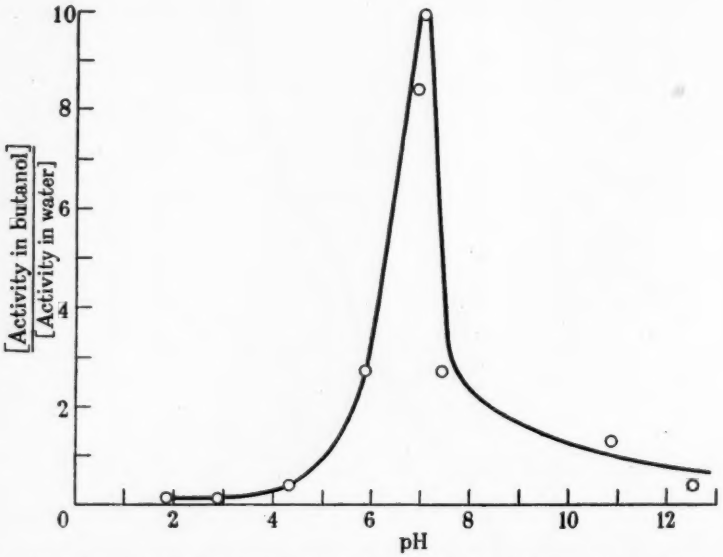

FIG. 5.—Variation with pH of the partition coefficient of ayfivin between *n*-butanol and water.

The partition coefficient of ayfivin (2 mg./ml.) between water and *n*-butanol varied greatly with the pH of the aqueous phase. Fig. 5 shows the results obtained by measuring the activity in the two phases. The relative solubility in butanol reached a maximum at the isoelectric point (pH 7·1), and fell sharply on either side of this value. The partition coefficient between water and amyl alcohol varied similarly with pH, though ayfivin was considerably less soluble in amyl alcohol than in butyl alcohol.

Precipitation and colour reactions.

Aqueous solutions of ayfivin gave amorphous precipitates with picric acid, picrolonic acid, Reinecke salt, mercuric chloride, and trichloracetic acid, and also on the addition of saturated ammonium sulphate or sodium chloride. They formed no precipitate with saturated aqueous solutions of benzoic or salicylic acid.

When benzoic acid or salicylic acid was precipitated in aqueous solutions of ayfivin very little activity was carried down on the precipitate.

Ayfivin gave a positive ninhydrin reaction. It gave no coloration with ferric chloride, or with alkaline nitroprusside in the presence or absence of cyanide. The Sakaguchi reaction for arginine and the Adamkiewicz reaction for tryptophane were negative. Knoop's reaction for histidine was positive.

Stability.

Ayfivin was stable for 10 minutes when heated at 100° C. in aqueous solution (1 mg./ml.) at pH 2, 7, or 10. It was stable in 2 N acid for 5 minutes at room

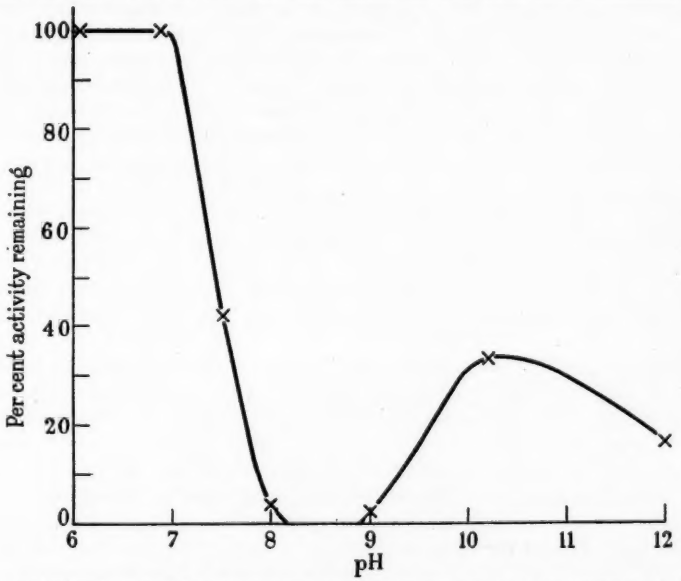

FIG. 6.—Inactivation of ayfivin in an aqueous solution (0·1 mg./ml.) at different pH values after 4 hours at 37° C.

temperature, but was inactivated when heated in 2 N acid at 100° C. for 5 minutes. It was largely inactivated when kept in N/10 sodium hydroxide for 4 hours at room temperature, or heated in N/100 sodium hydroxide for 15 minutes at 100° C. It was not inactivated by incubation with pepsin at pH 3, or with trypsin at pH 8.

Inactivation by copper ions.

In early experiments with purified ayfivin it was found that material kept at 37° C. for 4 hours in dilute aqueous solution (0·1 mg./ml.) showed a maximum instability between pH 8 and pH 9 (Fig. 6). Between pH 3 and pH 7 no activity was lost, and at pH 10–11 about 70 per cent of the activity was lost, while at pH 8–9 the loss of activity was almost complete. When dilute solutions of ayfivin at pH 8–9 were heated to 100° C. there was almost total loss of activity in 2 minutes

In contrast to the behaviour of purified material, the original culture fluid which had been adjusted to pH 8–9 showed no detectable loss of activity when kept for 4 hours at 37° C., or for 2 minutes at 100° C. The culture fluid therefore contained sustances that protected ayfivin from inactivation. It was found that the inactivation of purified ayfivin at pH 8–9 was largely or completely prevented by the following additions to the solution: commercial trypsin or pepsin (1 mg./ml.); horse serum (1 per cent); heart or lemco broth; glutamic acid, cysteine, or glycine (1 mg./ml.). Gelatine (1 mg./ml.) exerted some protective action, but was less effective than the other substances.

When the concentration of ayfivin was increased from 0·1 mg./ml. to 1 mg./ml. very little loss of activity was detected on keeping the solution at pH 8–9 for 4 hours at 37° C. The change in apparent stability with concentration suggested that the inactivation was caused by traces of an impurity in the solvent (laboratory distilled water). If the amount of ayfivin inactivated depended largely on the amount of the impurity, and thus on the volume of the solvent, the *proportion* of ayfivin inactivated would diminish as its concentration was increased.

It is now considered that a reaction of ayfivin with copper ions was responsible for this phenomenon. When the early experiments were later repeated no inactivation was found to occur. In the interval a new apparatus had been installed to supply distilled water. Inactivation occurred at pH 8–9 in the new distilled water, but not at pH 7 or below, when small amounts of copper sulphate were added to the solution. Table I shows the results obtained when ayfivin (0·1 mg./ml.) was incubated for 4 hours at pH 8·4 with varying amounts of cupric ions.

An amount of Cu^{++} as small as 0·25 μg./ml. caused substantial inactivation. Little, if any, inactivation was caused under similar conditions by a number of other heavy metal ions in concentrations approximately 10 times as large. The ions tested were Pb^{++}, Fe^{+++}, Zn^{++}, Co^{++}, Mn^{++}, Al^{+++}, Sn^{++}, and Hg^{++}. The loss of activity, which occurred in the presence of 2·5 μg. Cu^{++} per ml., was prevented by the addition of glutamic acid (1 mg./ml.) to the solution.

Attempts to restore the activity of ayfivin inactivated in the presence of cupric ions have not so far been successful.

TABLE I.

Cu++ μg./ml.	Per cent of activity remaining.
25·0	0
2·5	0
0·25	10
0·025	90
0·0025	100
0·00	100

Amino acids formed on acid hydrolysis.

When ayfivin was hydrolyzed for 24 hours with boiling 20 per cent hydrochloric acid almost all of its nitrogen appeared as α-amino nitrogen. The product showed a much stronger ninhydrin reaction than the original substance. When the ninhydrin reaction was carried out in the manner described by Moore and Stein (1948) the intensity of the colour shown by the hydrolysate from a given

weight of ayfivin hydrochloride was very close to that shown by the same weight of leucine. The hydrolysate gave a positive test for SH with nitroprusside in the presence, but not in the absence, of sodium cyanide.

Paper chromatography.

Using the method introduced by Consden, Gordon and Martin (1944), one-dimensional chromatograms on Whatman No. 1 paper were obtained from the ayfivin hydrolysate with the phenol-water system in the presence of HCN, with the phenol-water system in the presence of ammonia, and with the benzyl alcohol-water system. Chromatograms were also obtained from the acidic and from the basic plus neutral fractions of the hydrolysate which had been separated on Amberlite IR-4 by the method of Consden, Gordon and Martin (1948). The positions of the spots after development with ninhydrin indicated that the following amino acids were present: aspartic acid, glutamic acid, lysine, ornithine, histidine, cystine, phenylalanine, leucine and/or isoleucine. The amount of the leucines appeared to be larger than that of any other single amino acid.

Oxidation with D-amino acid oxidase.

The oxygen absorbed on treating ayfivin hydrolysate with a preparation of D-amino acid oxidase (Krebs, 1935) at 37° C. was measured in the Warburg manometric apparatus. The activity of the enzyme system was tested with DL-alanine as a substrate: the reaction was complete in 1 hour, and the oxygen taken up corresponded to the theoretical amount for the oxidation of the D-component. With ayfivin hydrolysate as a substrate the reaction appeared to be complete in 2 hours, and the amount of oxygen absorbed corresponded to 14 c.mm. per mg. of ayfivin hydrochloride. On this basis about 14 per cent of the nitrogen of the ayfivin hydrolysate was in the form of D-α-amino nitrogen.

DISCUSSION.

There can be little doubt that the main active constituent of ayfivin is a highly polar polypeptide. Although there is evidence that the most active preparations described in this paper are not homogeneous, their impurities appear to have physico-chemical properties and to contain amino acid radicals similar to those of the antibiotic itself. Even in the case of the crude product, the solubilities of the impurities were such that very little resolution occurred during an eight-tube counter-current distribution under acid conditions, and the material behaved as though it were largely a single compound. The fact that this material was quickly resolved into several components in a system at pH 7, and that some of the resulting fractions showed a considerable increase in specific activity, indicates that conclusions about the homogeneity of a polypeptide which are based on results from a restricted number of distributions in one system can easily be too optimistic. Craig, Mighton, Titus and Columbic (1948) have pointed out that the results of counter-current distributions, like those of all other methods, can only provide an answer to the question of the purity of a substance in terms of probability. With relatively large molecules several different criteria are desirable before the probability of a substance being homogeneous is considered to be very high.

The variation in the partition coefficient of ayfivin between butanol and water with changes in pH indicates that the active substance contains groups

that ionize over a wide range of hydrogen ion concentrations. The presence of such groups in the purified material is shown by the results of electrometric titration and of qualitative amino acid analyses. Anionic centres are provided by aspartic acid and glutamic acid, and cationic centres by histidine, ornithine and lysin. The hydrophobic side-chains of leucine and phenylalanine, which appear to be the main neutral amino-acids, would account in part for the high solubility of ayfivin in moist *n*-butanol at the isoelectric point. At this point the solubility may be increased by a displacement of equilibrium from the polypolar towards the non-charged form.

The electrometric titration of the purest preparation reveals a group which titrates in the region immediately on the alkaline side of the isoelectric point. A group in ayfivin, which ionizes in this region, may be concerned with the inactivation of the antibiotic that occurs under certain conditions in the presence of copper ions. The amount of inactivation is negligible at pH 7, but rapidly reaches a maximum at pH 8·5. A copper co-ordination complex is possibly involved in the reaction. The fact that there is a decrease in the amount of inactivation when the pH is raised from 8·5 to 10 would be understandable if a group inessential for antibacterial activity, either in ayfivin itself or in impurities, competed for copper at the higher pH. Competition by added amino acids and proteins has been shown to prevent inactivation.

An antibiotic named licheniformin, which is produced by a strain of *B. licheniformis*, has been studied by Callow, Glover and Hart (1947), and by Callow, Glover, Hart and Hills (1947). When grown under appropriate conditions, the strain A-5 of *B. licheniformis* also produces a licheniformin-like antibiotic (Hills *et al.*, 1949). Ayfivin and licheniformin differ markedly in chemical properties. Ayfivin, however, shows similarities to the antibiotic bacitracin, first described by Johnson, Anker and Meleney (1945). When the present work began little published information on the chemical properties of bacitracin was available, and it was doubtful whether the substance was a peptide (Anker, Johnson, Goldberg and Meleney, 1948). Both ayfivin and bacitracin could be extracted from culture fluid with *n*-butanol, but it was thought unlikely that they were identical because bacitracin was reported by Johnson, Anker, Scudi and Goldberg (1947) to be precipitated by benzoic acid and salicylic acid, and these reagents did not remove ayfivin from solution. A sample of commercial bacitracin, however, has now been found to behave like ayfivin towards benzoic and salicylic acid. The work of Barry, Gregory and Craig (1948) has established that purified preparations of bacitracin consist of polypeptide, and the amino acids they are reported to contain are very similar to the amino acids present in the most active preparations of ayfivin. Further investigations are required to establish the exact relationship between the two antibiotics.

Since this paper was written it has been shown that the inactivation in the presence of copper ions is accompanied by an uptake of oxygen and that it probably involves the oxidation of a suphur atom.

SUMMARY.

1. Preparations of ayfivin with high antibacterial activity were obtained from the culture fluid of the strain A-5 of *B. licheniformis*. The process of extraction involved adsorption of the active material on to charcoal, elution with a mixture

of *n*-butanol and dilute hydrochloric acid, transfer between butanol and water, and precipitation with picric acid. The crude product was further purified by 8-tube counter-current distributions in a system composed of amyl alcohol, *n*-butanol, and water or phosphate buffer at pH 7. No significant purification was obtained by 8-tube distributions in a system containing acetic acid at about pH 2·8.

2. The most active material behaved as a polypeptide with an isoelectric point at pH 7·1. It contained groups which ionized in the pH region 2–5, 5·5–8·5, and 10–12. On acid hydrolysis it yielded aspartic acid, glutamic acid, histidine, ornithine, lysine, cystine, phenylalanine, leucine and/or isoleucine.

3. The partition coefficient (ayfivin in *n*-butanol)/(ayfivin in water) varied rapidly with the pH in region of neutrality and was a maximum at the isoelectric point. On the alkaline side of the isoelectric point ayfivin was inactivated in the presence of copper ions, the amount of inactivation reaching a maximum at pH 8–9.

4. Ayfivin is quite different from licheniformin in its physical and chemical properties, but shows a similarity to bacitracin.

We are greatly indebted to Dr. D. W. Henderson and Mr. G. M. Hills of the Microbiological Research Department, Experimental Station, Porton, who supplied us with large amounts of culture fluid. Our thanks are also due to Miss R. Callow and Miss A. Pill for expert technical assistance.

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BIOLOGICAL OBSERVATIONS ON AYFIVIN.

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THE following paper records observations on the antibacterial, pharmacological and chemotherapeutic properties of impure ayfivin, the preparation and chemical properties of which have been described by Sharp, Arriagada, Newton and Abraham (1949).

EXPERIMENTAL.

Antibacterial Properties.

The activity of ayfivin against a number of species of bacteria was tested by the serial dilution method. Glass-filtered solutions of a crude preparation containing about 10 units/mg. were used for most of the experiments. This solution was diluted in a two-fold series and added in the proportion of 1 in 10 to tubes of heart extract broth, which were then inoculated with a uniform amount of culture of the organism under test. According to the hardiness of the organism the culture was used undiluted, or was diluted 1 in 10, 1 in 100, or 1 in 1000, so as to give a small inoculum, yet one that would be certain to grow. Modifications of the technique were made when necessary; for instance, 10 per cent serum was incorporated for the neisseria and some of the streptococci, and the tubercle bacillus was tested in Dubos' Tween and albumen medium. The clostridia were incubated in anaerobic jars.

Table I shows the results, in most cases after overnight incubation. Incubation for a further 24 hours had usually no effect on the titre, but occasionally doubled the concentration needed for complete inhibition.

Nine of the strains of *Staph. aureus* that were tested were relatively resistant to penicillin, requiring from 8 to 200 units/ml. for inhibition. These had the same sensitivity to ayfivin as the penicillin-sensitive strains.

The effect of ayfivin on two strains of *Leptospira icterohaemorrhagiae* in 12 per cent serum water was tested by a similar method.* In tests *in vitro* with this organism death rather than inhibition of multiplication is observed. Both strains were killed by a concentration of 1 in 10,000, higher dilutions having little or no effect.

Bactericidal action.

Dilution series that had been inoculated with *Str. pyogenes* and two strains of *Micrococcus* were plated out after 20 hours' incubation. There was no growth from the tubes containing the strongest concentrations; the smallest concen-

* We are indebted to Dr. J. A. H. Wylie for carrying out these tests.

tration that had killed ranged from the same as that which had inhibited growth to one 4 times greater.

There was no evidence that *Myco. tuberculosis* was killed by ayfivin.

TABLE I.—*Antibacterial Activity of Ayfivin. The figures in the third column show the concentrations of ayfivin in units/ml. which completely inhibited growth in liquid medium during* 20 *hours' incubation* (44 *hours in the case of the* Clostridia *and longer periods for* Myco. tuberculosis).

Species.	Number of strains.	Concentration inhibiting growth (units/ml.).
Staphylococcus aureus	18	0·125–>1·0
,, *albus*	1	1·0
Streptococcus pyogenes:		
Group A	5	0·015–0·063
,, B	1	>1·0
,, D and *Str. faecalis*	3	>1·0
,, G	1	0·5
Streptococcus viridans	5	0·25–1·0
,, *pneumoniae*, Types 1, 3, 6, 8, 19, and 2 strains not typed	8	0·031–0·5
Micrococcus lysodeikticus	1	0·031
,, *tetragenus*	1	1·0
,, sp.	1	0·015
Neisseria gonorrhoeae	1	0·25
,, *meningitidis*	2	0·5, 1·0
Corynebacterium diphtheriae var. *gravis, intermedius* and *mitis*	3	0·063–>1·0
Corynebacterium xerosis	1	0·008
Clostridium welchii	2	>1·0
,, *septicum*	1	>1·0
,, *oedematiens*	1	>1·0
,, *novyi*	2	>1·0
Bacillus anthracis	6	>1·0
Mycobacterium phlei	2	>1·0
,, *tuberculosis*,* bovine type	2	>2·0
,, ,, * human type	8	0·5–72
,, ,, * B.C.G.	1	0·5
Haemophilus influenzae	3	>1·0
,, *parainfluenzae*	2	>1·0
Pasteurella pestis	1	>1·0
,, sp.	1	>1·0
Salmonella typhi	2	>1·0
,, *enteritidis*	3	>1·0
Bacterium coli	2	>1·0
Pseudomonas pyocyanea	1	>1·0
Proteus	2	>1·0
Vibrio cholerae	2	>1·0

* We are indebted to Dr. A. Q. Wells for these determinations.

Effect of inoculum size.

With both *Staph. aureus* and *Str. pyogenes* alterations in the size of the inoculum had little effect on the titre, the greatest difference being a four-fold increase in the inhibiting concentration as the result of a thousand-fold increase in the size of the inoculum.

Effect of serum.

The presence of 50 per cent of serum had little or no effect on the activity of ayfivin. In liquid culture medium it increased the concentration needed to inhibit the growth of *Str. pyogenes* from 2 to 4 times, but this difference may have been associated with stronger growth of the organism in the enriched medium.

Solutions of ayfivin were also incubated for 4 hours with 50 per cent serum, and then diluted serially and tested on cylinder plates alongside a control solution that had been incubated with water. The plates were seeded with *C. xerosis*. There was no alteration in the size of the zone of inhibition as the result of the presence of serum.

Acquisition of resistance.

In vitro.—Using material containing 40–45 units/mg., *Staph. aureus* (National Collection of Type Cultures, No. 6571) was transferred from one dilution series to another 23 times in 6 weeks. Each transfer was made from the tube containing the highest concentration of ayfivin in the presence of which there was substantial growth. There was no increase of resistance at any time during the experiment.

Myco. tuberculosis, however, developed resistance to ayfivin after growth in its presence *in vitro*.*

In vivo.—Cultures of *Staph. aureus* recovered from mice that had been infected with that organism and treated with ayfivin for 4 days, the last dose being given from 3 to 10 days previously, showed no sign of increased resistance. The same was found after therapeutic experiments with *Str. pyogenes*.

Comparison of Ayfivin with Bacitracin by Serial Dilution Tests.

Towards the end of the work reported in this paper it became apparent that ayfivin was very similar to bacitracin, if not identical with it. There were some discrepancies between the reported sensitivity of certain anaerobic spore-forming organisms and Group B *Streptococcus* to bacitracin and those found for ayfivin. A direct comparison between ayfivin and bacitracin (kindly supplied by Dr. Meleney) by the serial dilution method already outlined disclosed no essential difference between the sensitivities of one strain each of *Cl. welchii*, *Cl. sporogenes*, *Streptococcus* Group A and *Streptococcus* Group B to bacitracin and ayfivin.

PHARMACOLOGICAL OBSERVATIONS.

Toxicity.

Acute toxicity to mice.

A comparison was made between the toxicity of single doses of two fractions obtained by the counter-current distribution method of Craig (1944). One

* For this observation we are indebted to Dr. A. Q. Wells.

fraction contained 30 units/mg. and the other 4 units/mg. Table II shows the mortality after intravenous injection into mice weighing 19 to 20 g.

TABLE II.—*Effect of Intravenous Injection of Two Fractions of Ayfivin.*

Units/mg. in sample.	Dose in 0·3 ml. saline.		Total number of mice.	Number of mice alive at the end of day—					
	Mg.	Units.		1.	2.	3.	4.	5.	29.
30	2·5	75·0	10	10	10	10	10	10	10
30	5·0	150·0	10	4	4	4	4	4	4
4	5·0	20·0	10	10	10	10	10	10	10
30	7·5	225·0	10	5	4	3	3	2	2
4	10	40·0	10	5	2	0	0	0	0

Table III shows the mortality after the subcutaneous injection of the same samples of ayfivin into mice weighing 20 g. It is apparent from these results that the fraction containing more ayfivin was more toxic than the less antibacterially potent fraction, which nevertheless contained toxic substances that were bacteriologically inert.

TABLE III.—*Effect of Subcutaneous Injection of Two Fractions of Ayfivin.*

Units/mg. in sample.	Dose in 0·6 ml. saline.		Total number of mice.	Number of mice alive at the end of day—			
	Mg.	Units.		1.	2.	3.	29.
30	10	300	10	10	7	5	5
4	10	40	10	10	10	10	10
30	20	600	10	10	1	1	1
4	20	80	10	9	4	2	2

The animals that died in a short time showed inco-ordinated movements before reaching a state of collapse.

Chronic toxicity to mice.

Mice weighing initially about 20 g. were injected with material containing 29 units per mg. Five mice received 5 mg. and 5 mice 10 mg. daily, divided into 4 doses, and given subcutaneously (a total of 1305 and 2610 units per mouse respectively) for 9 days. The animals gained in weight in the same way as control animals receiving subcutaneous injections of normal saline, and showed no sign of toxic reactions.

In another experiment material containing 32 units per mg. was used. Mice in 3 groups of 4 were given total doses of 48 mg. (1536 units), 24 mg. (768 units) and 12 mg. (384 units) respectively by subcutaneous injection in 6 days, four equal doses being given daily. In the group receiving the largest dose one mouse died and another became sick but recovered. The rest of the mice apparently suffered no ill effects from the drug.

In a therapeutic experiment to be described later 5 mice which received 40 mg. (1280 units) of similar material (32 units/mg.) in 4 days were in good condition at the end of 6 weeks.

Effect on the kidneys.

Histological evidence suggested that the death of mice receiving the larger repeated doses of ayfivin might be due at least in part to damage to the kidneys. Other organs appeared to be normal, but the condition of the kidneys was as follows :

After single intravenous doses of 5–7·5 mg. (of material containing 32 units/mg.) no kidney lesions were found when the animals were killed 3 and 17 days later.

After multiple small subcutaneous doses.—Two doses of 4 mg. (of the same material) were given subcutaneously on one day. The mouse was killed 3 days later. The epithelium of some of the tubules was much flattened, and the tubules throughout the kidney were filled with hyaline masses. There was little evidence of necrosis, but there were many mitoses of the tubule epithelium.

Another mouse received 5 doses of 4 mg. (of the same material) in 2 days, and was found dead 17 hours after the last dose. Here the hyaline material in places contained dead cells, and there was evidence of degeneration or necrosis of much of the epithelium. A mouse which received 9 consecutive doses of the same amount in 4 days survived, and was killed 17 days later. At this time the epithelium showed little sign of damage, but here and there in the superficial part of the cortex were small areas of activity where regeneration of the epithelium appeared to be in progress. Tubules in some of these areas were dilated to form small cysts.

After longer courses of smaller injections—2, 4 or 8 mg. of the same material daily for 6 days, 12, 24 or 48 mg. in all—there was no consistent evidence of damage, though kidneys from mice that were killed a few days after the last injection usually showed a few of the areas of regeneration described above.

In the therapeutic experiment to be mentioned, in which each of 5 mice received a total of 40 mg., the kidneys appeared to be normal when the animals were killed after 6 weeks.

There is no doubt that, like other antibacterial polypeptides, impure ayfivin in sufficient dosage sometimes severely damages the kidneys of mice, though the presence of lesions was not closely related to the number of units given.

Local effects on tissues.

No damage was caused to the skin of mice by the subcutaneous injection of solutions of ayfivin (30 units/mg.) containing 16·6 mg./ml. Five mice, given 2·0 mg. of material containing 32 units/mg. 5 times a day for 4 days in some of the therapeutic experiments to be described, showed no sign of damage in the skin or subcutaneous tissues when killed 6 weeks after the last injection, nor was damage to the skin noted in those dying during the therapeutic experiments.

Effect on the motility of human leucocytes and on red blood cells.

Human leucocytes were examined by the method of Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941). No deviation from control preparations was observed when the leucocytes were immersed in a concentration of 1 in 1000 ayfivin ; in a concentration of 1 in 500 all leucocytes appeared to be active at the end of 2½ hours, but thereafter they became more sluggish than

those in control preparations. In a concentration of 1 in 250 cells were active for about an hour, but thereafter became immobile.

The red blood cells in the preparations were unaffected by any of the above concentrations.

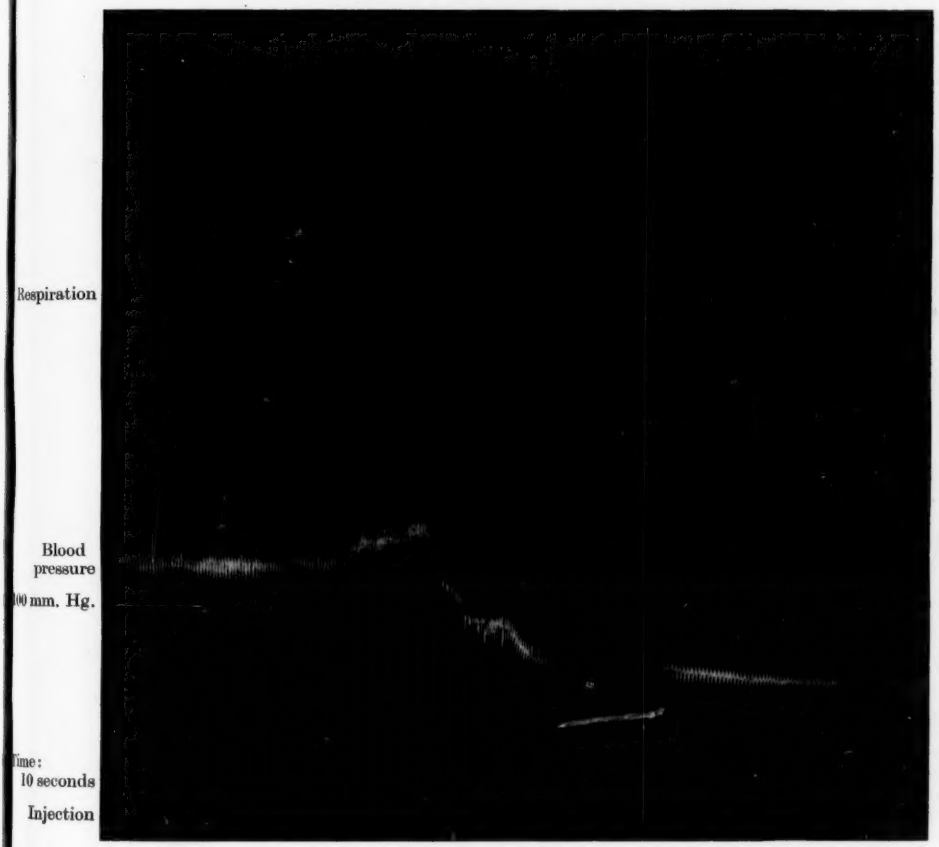

FIG. 1.—The effects on respiration and blood pressure of injecting 217·5 mg. of ayfivin intravenously into a decerebrate cat weighing 2·9 kg.

Effect on blood pressure and respiration.

Four experiments were made on cats anaesthetized with chloralose. The injection of 50 mg. of material containing 30 units per mg. into a cat weighing 3 kg. caused no fall of blood pressure ; a further injection of 100 mg. 14 minutes later caused a slow fall from 134 to 126 mm. Hg ; a further injection of 200 mg. caused a fall from 126 mm. to 72 mm. Hg in 14 minutes. The pressure gradually

returned to 126 mm. Hg during the next 2 hours. In the other animals similar results were obtained with smaller doses of less pure material.

Experiments were also done with cats decerebrated according to the method of Schmidt (1923). Fig. 1 illustrates the result of injecting a cat weighing 2·9 kg. with 75 mg./kg. (217·5 mg.) intravenously. There was a profound fall of blood pressure and stimulation of respiration. No recovery in the level of the blood pressure occurred. Doses of 25 and 50 mg. given intravenously to other decerebrate cats had no effect on the respiration or blood pressure.

It is clear that if a sufficiently large dose is given the substance causes a substantial fall of blood pressure.

Effect on smooth muscle.

The effects of ayfivin on smooth muscle were tested on guinea-pig uterus suspended in oxygenated Ringer-Locke's solution. A concentration of 1 in 5000 ayfivin (material containing 30 units/mg.) sometimes increased the amplitude of the rhythmic contractions; the effect was more marked with a concentration of 1 in 1250. After washing, the amplitude of the contractions became less again (Fig. 2). Sometimes no definite effect was observed.

Thus it is unlikely that the fall of blood pressure following intravenous injection into the cat is due to the presence of histamine-like substances in preparations of ayfivin.

Absorption and Excretion.

Absorption and excretion in mice.

It was found that ayfivin was absorbed after subcutaneous injection in mice and appeared in the urine. Use was made of its excretion by the kidneys to determine whether there was significant absorption from the alimentary canal.

Urine was collected from 2 mice weighing 20 g. 5 hours after the administration of 320 and 160 units by stomach-tube. The stomach and intestines were washed out with buffer solution and the washings were assayed. For comparison, similar observations were made on 2 mice that had received 32 units subcutaneously. Specimens were assayed by the cylinder-plate method, using *C. xerosis* as the test organism. Table IV records the results.

TABLE IV.

Mouse No.	Route.	Dose, units.	Total amount (units) recovered from urine.	Total amount (units) recovered from gastro-intestinal tract.
1	*Per os*	320	0	60
2	,,	320	0·45	27
3	,,	160	0·2	10·5
4	,,	160	0	13·5
5	Subcutaneous	32	16·5	0
6	,,	32	3	0·2

It is clear that little or no ayfivin is excreted in the urine following oral administration, and it may be inferred that the substance is not absorbed in significant amounts from the alimentary tract.

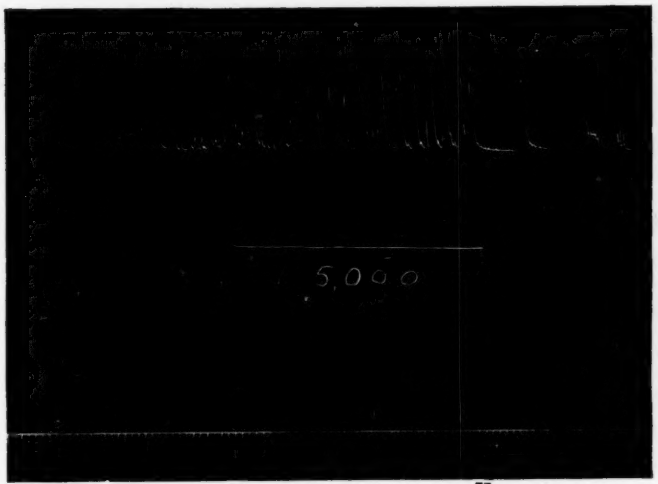

FIG. 2A.

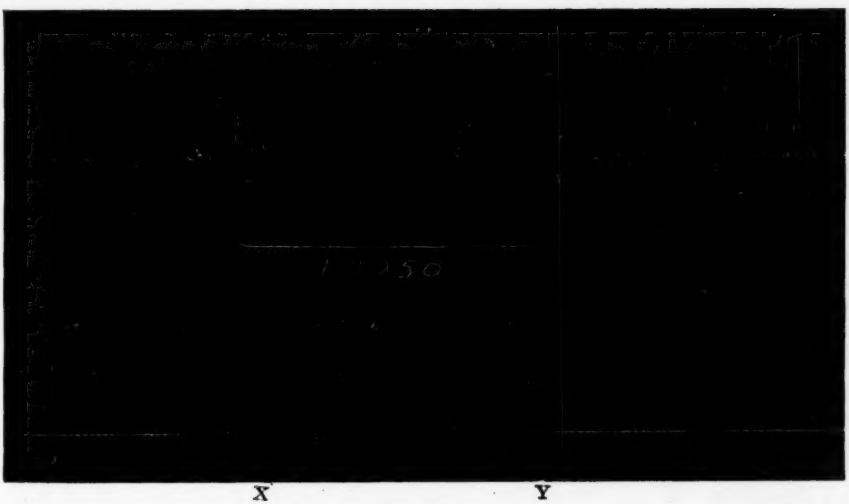

FIG. 2B.

FIG. 2.—A. At X, ayfivin added to bath to make a concentration of 1 in 5000. At Y, uterus washed with Ringer's solution. Time: 30 seconds. B. Similar to A, but concentrations 1 in 1250.

Blood levels and urinary excretion in rabbits.

Rabbits were injected subcutaneously with a sterile solution of ayfivin, and blood samples were withdrawn at intervals afterwards. Assays were performed on the serum by the micro-method described by Fleming (1944), using *Str. pyogenes* as test organism and red blood cells as indicator. The urine was collected in a metabolism cage and assayed by the cylinder-plate method, using *C. xerosis* as the test organism. Some of the results are shown in Fig. 3.

Animal Protection Experiments.

Against Str. pyogenes.

In 1947 the first experiments were done, using two groups of 10 mice which were infected by the intraperitoneal injection of 0·5 ml. of a 1 in 100 dilution of an overnight culture of the organism.

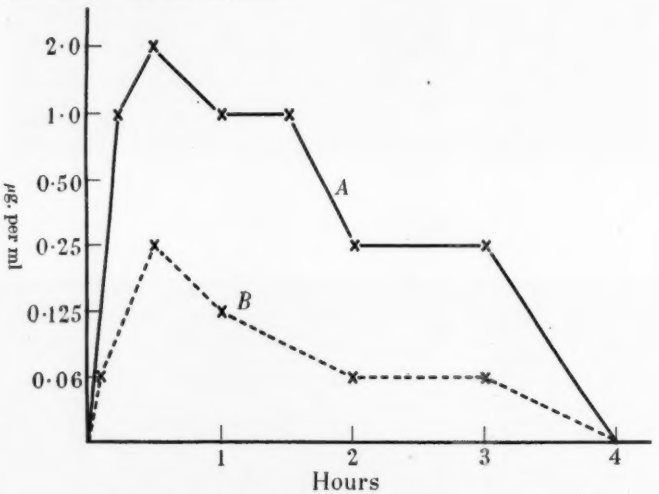

FIG. 3.—Concentrations of ayfivin in the blood serum of two rabbits after subcutaneous injection. A. ——— 30 mg. (960 units) injected. B.- - - - 10 mg. (320 units) injected. Total recovered in urine: A. 575 units, i.e. 60 per cent. of amount injected. B. 173 units, i.e. 45 per cent of amount injected.

A sample of ayfivin containing 10 units/mg. was used. In the first experiment a total of 9·5 mg. (95 units) was administered in divided doses 4 times daily for 2 days. The 10 control mice died within 36 hours, while the 10 treated mice were alive and well at the end of 10 days. In two other experiments conducted at this time it was found that even after as little as 0·5 mg. (5 units) given in 48 hours life was prolonged in all treated animals, and 1 mouse out of 5 was alive at the end of 8 days.

A more thorough repetition of these experiments was made in 1949, when it was again demonstrated that there was a chemotherapeutic effect (see Table V). *Str. pyogenes* (Wellcome CN 10), of which the lethal dose for mice was about 500 organisms, was used for infection. Its growth was inhibited *in vitro* by one part

TABLE V.—*Action of Ayfivin in Mice Infected with* Str. pyogenes.

Dose of ayfivin (units).		Total. number of mice.	Number of mice alive at end of day—							
Single.	Total (in 2 days).		1.	2.	3.	4.	5.	6.	7.	31.
15	150	5	—	—	—	—	—	—	5	5
7·5	75	5	—	5	4	3	—	—	3	3
3·75	37·5	5	—	5	3	2	—	—	2	2
1·88	18·75	5	—	5	2	—	—	—	1	1
0·95	9·5	5	5	2	1	—	—	—	1	1
No treatment (controls)		20	2	0						

of this preparation of ayfivin in 2·56 millions, that is by 0·012 units per ml. Mice weighing from 22 to 24 g. were injected intraperitoneally with 0·5 ml. of a 1 in 10,000 dilution of an overnight culture containing about 50,000 bacteria—that is, 100 lethal doses. Five groups of 5 mice each were treated with ayfivin given subcutaneously, and 20 untreated mice formed a control group. The preparation used contained 32 units per mg. On two successive days four injections were made at approximately 4-hour intervals, the first three injections on each day containing from 0·95 to 15 units for different groups, and the last containing a double dose. Even the smallest dose had some effect in prolonging life, but only the largest dose (150 units in all) gave complete protection. Streptococci were recovered from the heart blood of the animals dying during the experiment. The mice that survived gained weight, and when they were killed at 31 days post-mortem examination showed nothing abnormal.

Against Staph. aureus.

In 1947 experiments were performed with mice infected intraperitoneally with 1 ml. of a 1 in 5000 dilution of an overnight broth culture of *Staph. aureus* in 5 per cent mucin. Ayfivin containing 28 units per mg. was used for treatment. In the most successful experiment all of 7 treated mice survived in good condition for 1 month, after which they were not followed further. These mice received 3 doses of 2 mg. and 1 of 4 mg. daily, a total of 40 mg. (1120 units) being given in 4 days. In the same experiment mice receiving half this amount began to die on the 7th day, and gradually all died with localized staphylococcal abscesses.

In 1949 this investigation was repeated in more detail (Table VI). In Experiment 1, 45 mice weighing from 21 to 23 g. were used. An overnight serum broth culture of *Staph. aureus* (Wellcome CN 491), suspended in 5 per cent mucin to give a final dilution of the culture of 1 in 1000, was used to infect 45 mice, 0·5 ml. being injected intraperitoneally into each animal. This was equivalent to about 10 lethal doses. The plan of the experiment followed that for *Str. pyogenes*, and the same preparation of ayfivin was used, but larger doses were given, the staphylococcus being relatively more resistant by *in vitro* tests. The growth of strain CN 491 was inhibited by a concentration of 1 part of this preparation in 160,000, that is by 0·2 unit/ml.

Single doses ranged from 4 to 64 units for different groups, the spacing of the doses and the double dose in the evening being the same as for the streptococcus.

The earlier experiments had shown how hard it was to eradicate the staphylococcus completely, so treatment was continued for 4 days.

The 20 control mice were all dead with staphylococcal septicaemia within 36 hours. In the group of mice that received a total dose of 320 units two mice were killed by their companions between the 17th and 21st days. These showed no sign of staphylococcal infection, and the heart blood was sterile. All surviving treated mice were killed at the end of 6 weeks, at which time they appeared to be in good health, and had gained weight. On post-mortem examination 3 of the 5 mice that had received the smallest dose (total 80 units) were found to have staphylococcal abscesses in the liver and peritoneal cavity.

Table VI shows also the dosages and results in two subsequent experiments carried out in a similar way. In Experiment 2 the dose of staphylococcus culture in mucin was the same, but the organism had lost virulence, and the control animals were not all dead until the 15th day. In spite of the lower virulence, perhaps because the number of organisms used was the same, there was no reduction in the total dose of ayfivin needed to save life. By the time that Experiment 3 was performed the strain had recovered virulence about equal to that in Experiment 1. Ten times as many bacteria were injected (about 100 lethal doses), again in 5 per cent mucin, and this killed all control mice within 14 hours. Protection by the smaller doses of ayfivin was not so effective in this group.

Treated mice that survived for 6 weeks showed no sign of ill-health, and gained weight, but out of the total of 41 that survived and were killed at this time 8 had staphylococcal abscesses in the abdominal wall, in the mesentery, or

TABLE VI.—*Action of Ayfivin in Mice Infected with* Staph. aureus.

Experiment No.	Dose of culture diluted in 5 per cent mucin.[1]	Dose of ayfivin (units). Single.	Total (in 4 days)	Number of mice, total.	Number of mice alive at end of— Day. 1.	2.	3.	4.	5.	6.	7.	Week. 2.	3.	4.	5.	6.
1	0·5 ml. of 1 : 1000	64	1280	5	—	—	—	—	—	—	5	—	—	—	—	5
		32	640	5	—	—	—	—	—	—	5	—	—	—	—	5
		16	320	5	—	—	—	—	—	—	5	4[2]	3[2]	—	—	3
		8	160	5	—	—	—	—	—	—	5	—	—	—	—	5
		4	80	5	—	—	—	—	—	—	5	—	—	—	—	5[3]
	No treatment (controls)			20	3	0										
2	0·5 ml. of 1 : 1000	13	256	5	—	—	—	—	—	—	5	—	—	—	—	5[4]
		6·5	128	5	—	—	—	—	—	—	5	—	—	4	—	4[5]
		3·3	64	5	—	—	—	—	—	—	5	—	2	—	—	2
		1·6	32	5	—	—	—	—	—	—	5	3	2	—	—	2
		0·8	16	5	4	3	—	—	—	—	3	2	1	—	—	1[5]
	No treatment (controls)			10	6	5	—	—	3	—	3	1	0*			
3	0·5 ml. of 1 : 100	16	320	5	—	—	—	—	—	—	5	—	4	—	—	4[5]
		8	160	5	—	—	—	—	—	—	5	3	0			
		4	80	5	—	4	—	—	—	—	4	2	0			
		2	40	5	4	3	—	1	0							
		1	20	5	3	1	—	0								
	No treatment (controls)			10	0†											

[1] Virulence of staphylococcus fell at time of Experiment 2, and recovered afterwards.
[2] Killed by fighting.
[3] Three survivors had staphylococcal abscesses.
[4] Two survivors had staphylococcal abscesses.
[5] One survivor had staphylococcal abscesses.
* Last control mouse died on 15th day.
† All control mice dead within 14 hours.

in the liver. It was thus evident that the doses used did not eliminate staphylococci completely, except possibly in Experiment 1, in which the largest dose was 4 times as great as any dose used subsequently, and was near the toxic level.

Thus ayfivin, when administered in the proper dosage, can protect mice against certainly fatal infection with *Str. pyogenes* and *Staph. aureus*.

DISCUSSION.

It is clear from the data presented that ayfivin is one of those polypeptides which, under appropriate conditions, are capable of exerting a systemic therapeutic effect in mice, but which have the great disadvantage of causing severe damage to the kidneys in doses not much above those necessary for therapeutic effect.

The pharmacological properties and antibacterial range of ayfivin are very similar to those of bacitracin. During most of the time that this work was going on it was thought that there were differences between the two crude products, for some of the chemical and biological data originally published on bacitracin did not appear to accord with those on ayfivin, but a comparison recently made between crude ayfivin and crude bacitracin (such as is used for clinical work) has not so far revealed any chemical difference (Sharp *et al.*, 1949), nor has any biological difference been discovered. In the comparative antibacterial test reported here their range of activity was precisely the same. It is quite possible that ayfivin and bacitracin, though similar, are not identical, but this point may not be settled until the active polypeptides can be compared in a much more highly purified form.

SUMMARY.

Ayfivin, an impure polypeptide mixture obtained from a strain of *B. licheniformis*, has been shown to have powerful antibacterial properties and a low toxicity to mice. It has been shown to possess protective action against experimental infections in mice with *Str. pyogenes* and *Staph. aureus*. However, in doses not much greater than those required for therapeutic effect severe damage can be caused to the kidneys. Ayfivin appears to be closely related to bacitracin.

We are greatly indebted to Mr. J. Kent, Mr. H. Wheal, Miss M. Lancaster, Miss M. Bond and Mr. N. Smith for technical assistance.

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THE PREPARATION AND PROPERTIES OF ENZYMES FROM *CL. WELCHII* (TYPE B) FILTRATES WHICH DESTROY BLOOD-GROUP SUBSTANCES.

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SCHIFF (1935, 1939) first observed that cultures and culture filtrates from certain strains of *Clostridium welchii* possess the power to destroy the specific serological properties of the blood-group A-substance present in commercial peptone and human saliva. Schiff considered the enzyme to be specific for the A-substance and to be without action on the Group B factor in saliva.

Schiff and Weiler (1931*a*) observed that the normal faeces of persons of all groups and of certain animals contain an enzyme which readily inactivates the A and B blood-group substances. The enzyme is also present in saliva (Schiff and Weiler, 1931*b*), and is found in the saliva of secretors and non-secretors alike (Matson and Brady, 1936). The enzyme does not appear to be derived from the bacteria found in the mouth, and it has been suggested by Schiff and Burón (1935) that the enzyme is secreted by the glandular cells. Landsteiner and Chase (1935) reported that A-substance was decomposed by an organism, *Pullulomyxa botrytis* (Thaysen, 1939), which was isolated by Morgan and Thaysen (1933), and shown to be active in decomposing a number of specific bacterial polysaccharides.

The isolation of the specific blood-group substances of animal and human origin in a purified form led to a reinvestigation of the enzymic destruction of their serological properties (Morgan, 1946). A number of *Cl. welchii* (Type A) culture filtrates, crude and partially purified with reference to collagenase (κ-toxin), an enzyme which breaks down collagen (Oakley, Warrack and van Heyningen, 1946 ; Bidwell and van Heyningen, 1948 ; Bidwell, 1949), were examined, and were found to contain enzymes which would rapidly inactivate the purified A, B and H blood-group substances. Increase in activity of the collagenase, hyaluronidase and certain toxins common to these filtrates during their purification and concentration, however, was not correlated with increased capacity to inactivate the group mucoids, and it appeared that they were not the factors responsible for the decomposition of the blood-group substances. Nor does the λ-enzyme of Oakley, Warrack and Warren (1948) appear to be involved, since after purification of a *Cl. welchii* (Type B) culture filtrate with a resultant 50 per cent yield of this enzyme, only 2 per cent of the A-enzyme remained. Heating the culture filtrates for 1 hour at 56° resulted in the inactivation of the enzymes which destroy the A- and B-substances, but left unimpaired the enzyme which decomposed the so-called O-substance, now referred to as H-substance (Morgan and Watkins, 1948). The results of these investigations indicate that at least two enzymic activities are displayed by the partially purified culture filtrates.

The activity which destroys the serological properties of the A and B group substances is thermolabile; the other enzymic activity is relatively thermostable and destroys only the H-substance.

The examination of culture filtrates from selected strains of *Cl. welchii* (Type B) has shown that enzyme preparations of much greater activity can be obtained readily, and the work described in this communication concerns the purification and properties of the group specific enzyme preparations obtained from Type B strains. The intended separation of the enzymic activities has not been achieved, and therefore the A- or B-enzymes cannot yet be obtained free from H-enzymic activity. A preliminary account of this work has already appeared (Stack and Morgan, 1948).

EXPERIMENTAL.

Materials and Methods.

Cultures.

The *Cl. welchii* (Type B) strain used by Schiff (1939), also strains CN 1253 and CN 1990, were found to yield the most active filtrates encountered. Fourteen other strains examined after two subcultures on peptone medium were found to yield not more than one-fifth of the enzyme titre found in the culture filtrates of the three chosen strains. Another strain, originally producing less enzyme than these, produced filtrates of maximum activity after three subcultures had been made. Culture filtrates from representative Type A, C and D strains contained about one-tenth of the enzyme activity found for average Type B strains. Tests on culture filtrates from a few strains of *Cl. histolyticum*, *B. anthracis*, *Staph. aureus*, *Ps. pyocyanea* and *V. cholerae* failed to reveal the presence of enzymes able to inactivate the blood-group substances.

The organisms were grown on a peptone medium consisting of Evans peptone, 3 per cent, and sodium β-glycerophosphate, 2·5 per cent, supplemented before inoculation with glucose, 2 ml. per 100 ml. of a 10 per cent solution, and thiolacetic acid, 2 ml. per 100 ml. of a 0·4 per cent. solution. Without glucose, enzyme yields were generally one-third less, but without thiolacetate, only one quarter of the enzyme production occurred. Glucose caused no stimulation of enzyme production unless thiolacetate was present. The addition of 0·01–0·20 per cent of the test substrate caused no adaptive response. As group substances are present in the peptone used in the medium, the lack of adaptive response was confirmed by growing the same strain in the simplified medium described by Rogers (1945) with or without the addition of purified pig gastric mucin (A- and H-substances).

Specific blood-group substrates.

The test-substrate employed, unless otherwise stated, was a purified mucoid material obtained from a commercial preparation of pig gastric mucin according to the method described by Morgan and King (1943). The substance possessed A and H, the so-called O, activity. Recent work has shown that the O specificity of the mucoid is not the same as would be expected if the material were a product of the *O* gene. For this reason Morgan and Watkins (1948) suggested that the so-called O character would be better designated by the letter H, as this symbol would serve to emphasize that it is not a product of the *O* gene, but that it is a

heterogenetic material common to the great majority of human erythrocytes, irrespective of their A B O Group.

In a few experiments a preparation of human A-substance was employed which showed no H-activity, and on the basis of its chemical, physical and immunological properties was most probably homogeneous. Similarly, carefully purified H-substance obtained from pseudomucinous ovarian cyst fluids was used in other experiments. A potent specimen of ovarian cyst fluid obtained from a Group B secretor served as a source of B-substance. These materials were readily inactivated by the enzyme preparations.

For convenience in recording the results the enzymic activities which destroy the A-, B- and H-substrates are assumed to be due to separate enzymes and are termed A-enzyme, B-enzyme and H-enzyme respectively. The properties of the B-enzyme have not been examined in detail, but in those examined they do not appear to be distinguishable from those of the A-enzyme, except in their group specificity.

Measurement of enzymic activity.

The amount of the specific substrate, present at any given time after mixing with the enzyme preparation, was determined by means of the agglutination inhibition test described earlier (Morgan and van Heyningen, 1944). The anti-A, anti-B and anti-H sera used throughout the investigation were frequently titrated in inhibition tests against " standard " preparations of A-, B- and H-substances, respectively. The sera were stored in 2 to 3 ml. amounts without antiseptic and in sealed ampoules at $-10°$.

Ideally, the amount of change taking place over a short interval of time and in the presence of a large excess of unchanged substrate should be measured to determine the activity of an enzyme preparation. In this instance, however, measurements of this kind cannot be made owing to the insensitive nature of the agglutination inhibition test, which measures the excess antibody left in the system after the undecomposed group substance has neutralized a part of the standard dose of agglutinin. Agglutinin and, therefore, substrate concentrations differing by 50 per cent can readily be determined, and differences in concentration of this order are usually employed in agglutinin titration tests. If care is taken and all dilutions are made with graduated pipettes in place of the Pasteur pipettes usually employed in this type of titration, differences of 33 per cent in agglutinin concentration can be detected. For the simple purpose of following the change in activity of an enzyme preparation, for example during purification, 50 per cent differences, as derived by making progressively double dilutions of the enzyme-substrate system, are convenient, and have been used in this investigation.

A unit of enzymic activity is defined as that amount of activity which will bring about an extent of inactivation of 1·0 ml. of 0·10 per cent substrate in 2 hours at 37° and pH 7·0, which results in the lowering of the inhibition titre by four tubes when the usual two-fold geometrical dilution scale is employed. This unit is equivalent to the destruction of 94 per cent of the serological activity of 1·0 ml. of a 0·10 per cent solution of the blood-group substance.

Tests for the measurement of enzymic activity were carried out as follows: The group substance, 1·0 ml. of a 0·10 per cent solution, was mixed with different amounts of the enzyme preparation contained in 1·0 ml. of McIlvaine buffer, pH 6·8–7·0. The mixture was incubated at 37° for 2 hours in the presence of toluene

and then heated for 10 min. at 100°. The latter step is essential, otherwise the enzyme preparation brings about a sensitization and false agglutination of the red cells used in the agglutination-inhibition test subsequently employed to measure the extent of inactivation of the substrate.

The results of a typical titration carried out to determine the activity of an enzyme preparation are set out in Table I, from which it will be seen that 0·80 mg. of the enzymic material brought about a lowering of the inhibition end-point by four tubes, i.e. decomposed 94 per cent of the substrate under the standard test conditions. By definition, this amount, 0·80 mg., of the preparation contains one unit of activity. With the same laboratory "standard" a parallel series of results was obtained for the H-enzyme tests.

TABLE I.—*Showing the Inactivation of A-substance by Enzyme in 2 Hours at 37°, pH 7·0.*

Enzyme (mg. per ml.).	Inhibition of agglutination of A-cells by anti-A serum after the addition of the following dilutions of 0·10 per cent substrate:									Residual substrate (per cent).	Calculated A-enzyme (units/mg.).
	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256	1 : 512.		
0	0	0	0	0	0	0	0	1	2	100	—
0·2	0	0	0	0	0	0	1	2	3	50	(1·2)
0·4	0	0	0	0	0	1	2	3	4	25	1·2
0·6	0	0	0	0	1	2	3	4	4	12	1·2
0·8	0	0	0	*1*	2	3	4	4	4	*6*	*1·2*
1·0	0	0	1	2	2	3	4	4	4	3	1·2
1·2	0	1	2	3	4	4	4	4	4	1·6	1·2
1·6	1	2	3	3	4	4	4	4	4	0·8	1·1

0 = Absence of agglutination; 1 = groups of a few cells (the end-point); 2 = larger groups, many free cells; 3 = clumps visible without magnification; 4 = macroscopic agglutination.

In view of the insensitive nature of the agglutination inhibition test it is advisable to determine as closely as possible the amount of enzyme which will bring about a lowering by four tubes of the inhibition end-point of the standard substrate, measured under the defined conditions. When many determinations of activity are involved, however, as is inevitable for example in following the efficiency of purification procedures, greater or less inactivation than 94 per cent is frequently recorded, and an empirical interpretation of the end-point titres given in Table I has been used for the determination of the activity of the preparations in terms of the proposed units.

It has been found that if the difference in the number (N) of two-fold dilutions required to give the agglutination inhibition end-point before and after the enzyme has acted, be multiplied by the initial quantity of substrate (1·0 mg.) and divided by the amount of enzyme used, the result is approximately constant. The above definition of the unit employed can therefore be expressed in the working formula:

$$\text{Enzyme units} \frac{\text{N}}{4} \times \frac{\text{Initial substrate}}{\text{Initial enzyme}}$$

Table I contains the titration results obtained in one particular experiment in which the enzyme-substrate ratio was varied 5-fold. The calculated activity in units is in each instance the same within the experimental error. Similar

results were obtained when the H-enzyme and its substrate were examined. The extent of substrate destruction by an appropriate amount of enzyme was not affected by ten-fold dilution of the system before incubation or by employing an acetate buffer.

Enzyme Production during Growth of Cl. welchii (*Type B*).

Culture filtrates show maximum activity as soon as vigorous gas evolution ceases, usually about 12 to 15 hours after inoculation. The A-enzyme reaches its maximum titre at this time and then declines, whereas the H-enzyme, which is more stable under these conditions, remains at this optimal level for at least another day. The results of numerous experiments showed that the production of the enzyme occurs equally well whether the medium fills small or large vessels. The best yields are obtained when a 1 per cent inoculum from a vigorously growing subculture is added to the warm medium, freshly supplemented with glucose and thiolacetic acid. Poorer yields of A-enzyme, which, however, showed greater specific activity, could be obtained by growing the organisms in peptone medium contained in a cellophane dialysis sac, surrounded by fresh medium at 37°.

Stability of Enzyme Solutions.

Cultures were centrifuged and the clear supernatant fluids stored at 0 to 2° under toluene. If used within a day or so, the supernatant fluids show good enzymic activity against A-, B- and H-substances. If required for use more than a week later storage at − 10° without preservative is essential, but not always successful for the A-enzyme. The H-enzyme is fairly stable in aqueous solution at 0°. Drying a fresh culture filtrate from the frozen state prevents rapid deterioration of the enzymes. Precipitation of the enzymes by the addition of solid ammonium sulphate to the culture medium gives rise to a sticky scum, which partially dissolves in 80 per cent of glycerol to yield a stable enzyme solution. The addition of 0·4 per cent of gelatin, gum arabic, or peptone, does not stabilize aqueous solutions of the A- and B-enzymes. The stability of the enzymes was examined at pH levels between 3 and 12 by incubating for an hour at 20° before readjusting to pH 7·0 and testing. It was found that the A- and H-enzymes retained their full activity under these conditions from pH 5 up to a value as high as pH 11.

In confirmation of the earlier results of Morgan (1946), it was found that incubation in the absence of substrate for one hour at 56° destroys the A- and B-enzymes and such heated preparations can be used for control purposes. Fig. 1 shows that the A- and H-enzymes can be qualitatively differentiated in the one direction by heating at 55° for 10 minutes owing to the relative stability of the H-enzyme in buffer (pH 7·0) at this temperature. A preliminary incubation of the enzyme-substrate mixture at 55° or 60° for 10 minutes prior to the usual 2-hour reaction period at 37° reveals that the A-enzyme is protected at 55° by its substrate, but not at 60°, while the H-enzyme is protected at 60°.

If, after the usual enzyme-substrate incubation for 2 hours at 37°, a further 1·0 mg. amount of substrate be added, it has been found that on repeating the incubation, complete inactivation of the additional H-substance by the H-enzyme occurs. However, the A-enzyme is unable to inactivate the extra A-substrate completely—a finding which is not unexpected in view of the more labile character

of the A-enzyme, which presumably loses the protection of its substrate towards the end of the first incubation period.

Unit quantities of the purified enzymes in phosphate buffer were incubated for 2 hours at 37° with equal volumes of 0·02 M solutions of the following substances: Potassium cyanide, formaldehyde, glycerol, ascorbic acid, phenol, manganous chloride, ferric chloride, lead acetate, and silver nitrate. After further incubation with substrate, the H-enzyme titre was found to be enhanced by the first and depressed by the last three substances. Inhibition only occurred in tests where precipitation of the metal phosphates occurred. The serological test was not affected in blank experiments by the substances which appeared to interfere with the enzyme. These results are similar to those reported by Schiff and Weiler (1931*b*) and by Stimpfl (1932).

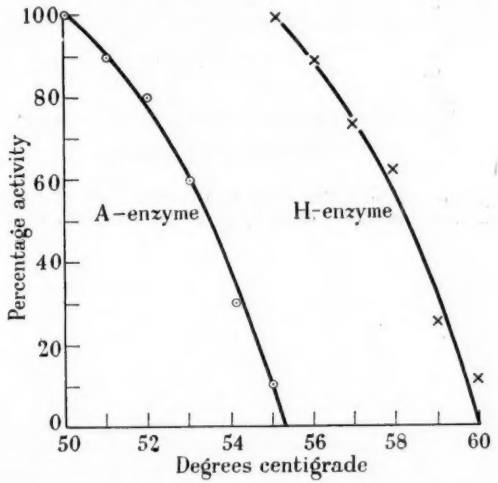

FIG. 1.—Differential inactivation of A- and H- enzymes by incubation for 10 minutes over the range 50–60°.

Optimum Temperature and pH of Reaction.

The optimum temperature for the reaction between the A- and H-enzymes and their substrates was investigated by incubation at pH 7 for 2 hours. The results indicated that both enzymes are most active over the range 40–45°, and still appreciably inactivate their substrates at temperatures above 50°. Fig. 2 is a composite curve based on over fifty readings averaged over 5° temperature intervals in order to detect the small differences in activity observed between 30° and 50°, measurements on both A- and H-enzymes being combined, since there were no observable differences between them. This conclusion is not at variance with the evidence on temperature stability, for in the latter experiment no substrate was present.

The rate of enzyme action at 37° was approximately doubled at 47° and halved at 27°. The results set out in Fig. 3 show that purified enzymes can be assayed in 10 minutes instead of the 2 hours required for culture filtrates. Inactivation

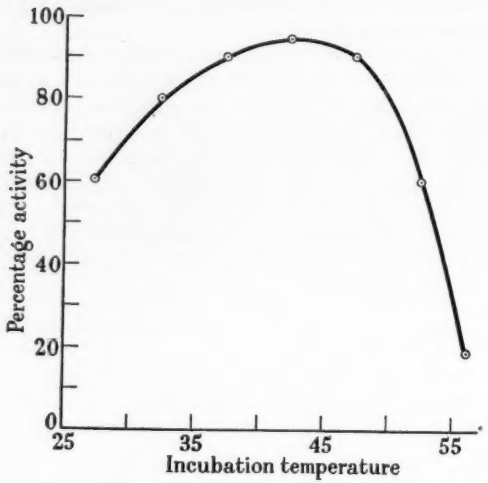

FIG. 2.—The variation of enzyme activity with temperature. Showing the mean values over 5° ranges. Extreme values differ by ± 20 per cent from those plotted.

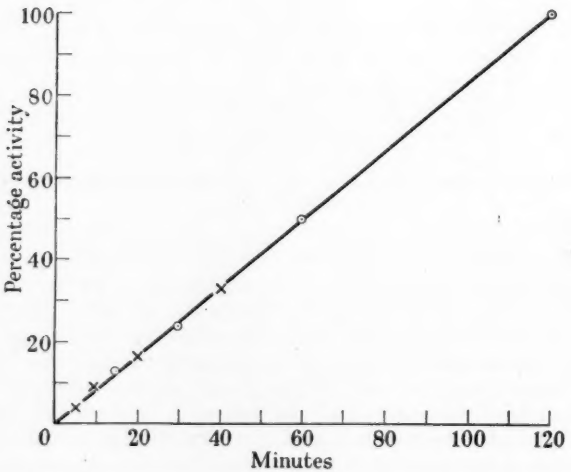

FIG. 3.—Showing the relationship between incubation period and enzyme activity calculated from (*a*) the enzyme-substrate ratio, which was progressively halved as the incubation period was doubled, and (*b*) the constant inactivation of the substrate resulting from this procedure.

of the substrate to the same extent was obtained by keeping constant the product of enzyme concentration and incubation period.

To determine the influence of pH on the activity of the enzymes a standard enzyme preparation was mixed with 0·10 per cent substrate in the presence of citrate, phosphate and borate buffers to give pH values between 3·7 and 9·8. The mixtures were incubated at 37° for 2 hours, readjusted to pH 7·0 and heated at 100° for 10 minutes before titration for undecomposed blood-group substance. The averaged results of a number of experiments compose the curves given in Fig. 4, from which it will be seen that there is a steady increase in A-enzyme activity from pH 3·7 to about pH 5·5. The activity then falls until at pH values

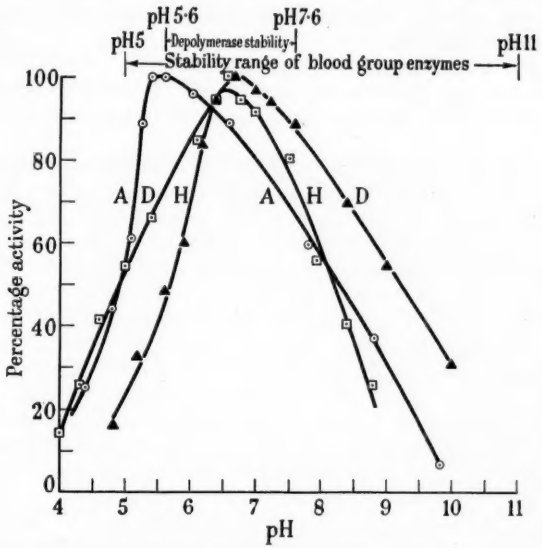

FIG. 4.—The variation of enzyme activity between pH 4 and 10. A (⊙——⊙) A-enzyme : H (▲——▲) H-enzyme ; D (⊡——⊡) depolymerase.

above 9 no significant inactivation of the specific substrate occurs. The H-enzyme behaves similarly, but shows its optimum activity at about pH 6·5. In both instances, however, over 90 per cent of the maximum activity is shown over a range of 1·2 pH units. In following the isolation and purification of the enzymes which will now be described, the measurement of enzymic activity has been carried out within the pH range 6·8–7·0, which provided a favourable condition for enzyme action, destruction of residual enzyme, and titration of residual substrate.

The Purification of the Blood-Group Enzymes.

The assessment of the specific activity of materials obtained as a result of fractionation of crude culture supernatant fluids was made by expressing the activity in terms of mg. " tyrosine " or mg. of nitrogen. " Tyrosine " was deter-

mined by means of a photo-electric colorimeter after the addition of sodium carbonate and Folin and Ciocalteu's reagent (1927) to samples containing 20–40 μg. " tyrosine " (light filter : Ilford 204). Nitrogen was similarly determined with Nessler's reagent (light filter : Ilford 302) after samples of the enzyme preparations, containing 20–40 μg. N, had been digested with H_2SO_4 and H_2O_2, using a procedure similar to that described by Miller and Miller (1948). The more convenient estimation of " tyrosine " was permissible, since the quantity of nitrogen present in purified material was consistently 3·7–3·8 times greater than the " tyrosine " content.

The addition of 70 g. per cent $(NH_4)_2SO_4$ to fresh culture supernatant fluids gives rise to protein scums which are partly soluble in aqueous buffer at pH 7. By this means the specific activity is increased about eight-fold for a 30 per cent recovery of the enzymes.

Further similar experiments demonstrate that the most active material, 50 H-enzyme units per mg. tyrosine, is thrown out of solution between 40 and 43 per cent added $(NH_4)_2SO_4$, but this fraction accounts for only a small part of the total activity.

Attempts to remove impurities from crude enzyme preparations by simple dialysis were without success owing to the extensive loss of enzymic activity. Ultrafiltration by means of dialysis sacs, strengthened by silk jackets and kept under reduced external pressure, reduced the fluid to one-tenth of its original volume. A considerable degree of purification is achieved by this means, but again the total activity recovered indicates that an extensive loss occurs.

Shaking culture filtrates or partially purified material in McIlvaine buffer at pH 7 with alumina, bentonite, calcium phosphate, Celite, Decalso, Fuller's earth, kaolin and magnesium silicate failed to bring about a separation of the enzymes or to remove useful amounts of impurities. Passage of the mixed enzymes through columns of charcoal-glass powder mixtures usually resulted in the adsorption of at least 50 per cent of the A-enzyme activity, together with 30 per cent or so of the total solids in the preparation. Passage through columns of alumina or calcium phosphate, however, allowed half the H-enzyme to be recovered in the filtrate with very little A-enzyme. On no occasion was there evidence that the adsorbed enzymes could be eluted by altering the pH or by running moderate concentrations of electrolytes through the columns.

Attempts to adsorb the enzymes on charcoal in the presence of 3M $(NH_4)_2SO_4$ were more successful, for subsequent elution from the charcoal with 1M $(NH_4)_2SO_4$ or buffer at pH 7·0 gave satisfactory yields of enzymes of considerably enhanced purity. For example, 1·0 g. of dried crude culture filtrate was dissolved in 20 ml. 3M $(NH_4)_2SO_4$ and shaken gently with 0·40 g. charcoal. The charcoal was recovered and eluted successively with 2M $(NH_4)_2SO_4$, 1M $(NH_4)_2SO_4$ and water. As 1M $(NH_4)_2SO_4$ appeared to have only a slight influence on the agglutination inhibition test, the two $(NH_4)_2SO_4$ eluates were reduced below this salt concentration by brief dialysis at 0–3°. The behaviour of the enzyme dialysed after exposure to similar concentrations of $(NH_4)_2SO_4$ in the absence of charcoal served as a control. There is an increase in the specific activity of the control material as a direct result of simple dialysis. The results show that when crude enzyme preparations which possess one H-unit associated with one mg. " tyrosine " are adsorbed in the presence of 3M $(NH_4)_2SO_4$ and eluted by 1M $(NH_4)_2SO_4$, half of the enzyme is recovered in the eluate and the material so obtained then shows

about 40 H-units per mg. " tyrosine." Table II indicates the degree of duplication obtained in consecutive experiments, the pairs of figures for specific activities showing good agreement.

TABLE II.—*Showing the Activity of Enzyme Preparations Eluted from Charcoal by Reducing the Ammonium Sulphate Concentration.*

Fraction tested.	Ammonium sulphate (M).	H-units per cent.	H-units/mg. tyrosine.
Original	0	(100)	1 ; 1
Control	3	60 ; 70	3 ; 3
Eluate A	2	6 ; 3	5 ; 4
Eluate B	1	38 ; 23	40 ; 40
Eluate C	0	6 ; 4	7 ; 8
Filtrate	3	10 ; 15	1 ; 1
SUM	—	60 ; 45	3 ; 3

The addition of 3 volumes of acetone to the crude filtrates as suggested by Schiff (1939), who employed a different medium, resulted in the deposition of an oily layer without giving rise to a significant increase in the specific activity of the material thrown out of solution. Treatment of fresh culture filtrates with an equal volume of acetone at 0° gave a satisfactory increase in purity with a yield of 70 to 80 per cent of the original activity. Methanol and ethanol have been employed with similar results. This method was used to prepare a quantity of the mixed enzymes suitable as a laboratory " standard." The details for preparing a useful quantity of enzymic material by this process are as follows :

Conical flasks filled nearly to the neck with 950 ml. of sterile peptone medium were inoculated and incubated overnight at 37°. " Superaid," 0·5 per cent, was added and the culture fluid filtered through paper. After cooling in an ice-salt bath the filtrate was mixed with an equal volume of acetone which had been cooled to − 30°. Regulation of the rate of acetone addition during the 10–15 min. required to complete the process ensured that the precipitation took place at 0°. Material thrown out of solution was collected by centrifugation and extracted with 25 ml. of cold McIlvaine buffer at pH 7·0. In six such experiments the average yield of the enzymes, purified some 150 times with respect to nitrogen or " tyrosine," per unit of activity, was not less than 60 per cent. Further extraction of the residue with buffer after the first treatment yielded additional material of lower specific activity. The main extracts were immediately shell-frozen in large round flasks and dried at 0·1 mm. pressure from the frozen state.

TABLE III.—*Showing the Activity of Acetone-Precipitated Blood Group Enzymes.*

Original (Av. A and H units/g.).	A-enzyme (units/g.).	H-enzyme (units/g.).	Depolymerase* (units/g.).	Nitrogen (mg./g.).
25	26†	24†	11†	70
25	1700	1200	450	31
36	1600	1600	640	30
48	2600	2400	440	22
58	2900	2900	500	18

* After storage for 3 months.
† Crude filtrate not acetone precipitated.

Thorough drying *in vacuo* over P_2O_5 of the white powders so obtained was followed by transfer to Macartney bottles at 0°. It was noticed, however, that not more than half the initial activity remained after two months' storage under these conditions. Details of the acetone precipitation of 4 culture filtrates are set out in Table III. The corresponding figures for a crude filtrate are also included. A carefully purified preparation contains at least 120 units of A- and H-activity per mg. nitrogen. Reprecipitation of material showing this order of activity with 0·4–1·3 volumes of acetone under the above conditions did not significantly increase the purity.

Immunization of Rabbits with Enzyme Preparations.

As a result of the failure to obtain preparations of A-enzyme devoid of power to inactivate H-substance by the methods described, an attempt was made to immunize rabbits with H-enzyme with the hope that an anti-H-enzyme would be produced which could be used to block the specific activity of H-enzyme without influencing the A-activity of the preparation. To this end rabbits were immunized with the unheated enzyme and with preparations heated at 56° for 10 min., which showed considerable specific A- and H-, and H-enzymic activity respectively.

The toxic nature of the material injected caused the death of four of the experimental animals, but one animal in each of the two groups survived the immunization and received a total of 1·6 mg. of enzyme protein over a period of 4 weeks. The natural A- and O-agglutinins were removed from the rabbit sera and suitable amounts of the sera were included in the standard test for enzyme activity. The results are given in Table IV, and show that on mixing the rabbit serum with the enzyme before the addition of substrate, some of the enzyme was neutralized or inactivated, for less enzymic activity was found than was added. Normal serum had no such effect.

TABLE IV.—*Showing the Neutralization of Enzyme by the Serum of Rabbits Immunized with* (a) *Heated and* (b) *Unheated Enzyme Preparations.*

Antigen (enzyme).	Antiserum used (ml.).	Units present.	Units found.
Heated	0·50	1·0A	0
	0·50	1·5A	0·5
	0·25	1·5A	1·0
	0·50	1·7H	0
Unheated	0·50	1·5A	0
	0·25	1·5A	0·8
	0·12	1·5A	1·2
	0·50	1·7H	0

The Enzymic Depolymerization of Pig Gastric Mucoid.

Specimens of mucoid isolated from commercial hog gastric mucin frequently show a high viscosity, and it has been observed that during the enzymic inactivation of the A and H serological properties of this material, a rapid fall in viscosity occurs. In our experience specimens of A- or H-mucoid isolated from individual pig stomach linings by peptic autolysis, and the A- and H-substances recovered from human ovarian cyst fluids, show no significant viscosity. Artificial

mixtures of A- and H-substances which individually show low viscosity are also non-viscous. It would seem, therefore, that a component of the commercial mucoid which is neither A- nor H-substance is responsible for the high viscosity of this material. It is of interest that the " depolymerase " present shows many properties which are similar to those of the group enzymes, and has been found to accompany these enzymes to the same extent after they have been purified as much as two hundred-fold in terms of the A- and H-enzyme activities (Table III). The highly viscous substrate is not depolymerized by hyaluronidase, and solutions of hyaluronic acid are not rendered less viscous by the blood-group enzyme preparations. Unlike hyaluronidase, the *Cl. welchii* depolymerase does not require optimum salt and buffer concentrations. The behaviour of buffered solutions of the depolymerase in the presence of the mucoid was followed in Ostwald pattern viscosimeters over a period of about 30 minutes at 37°. During this time a typical *Cl. welchii* (Type B) filtrate will reduce the relative viscosity of an equal volume of 0·25 per cent gastric mucoid from 1·6 to 1·3. The amounts of enzyme and substrate chosen were governed by the fact that at substrate concentrations greater than 0·25 per cent, the relationship between flow time and substrate concentration in the absence of enzyme ceased to be linear. The quantity of depolymerase present in the *Cl. welchii* culture filtrates decreased steadily after reaching a maximum at the end of 24 hours' growth. The pH-activity curve for this enzyme and the pH-stability range, which is considerably narrower than that of the blood-group enzymes, are shown in Fig. 4. Behaviour similar to that of the blood-group enzymes was noted on adsorption and precipitation, e.g. one-third of the depolymerase was recovered from culture filtrates by adding 70 per cent ammonium sulphate, the specific activity being increased 8 times, whereas the acetone purification procedure described above improved the specific activity some two hundred times. Hydrogen peroxide, potassium cyanide, and ascorbic acid, 0·02 M, caused some inhibition of activity. Toluene and merthiolate could safely be used as preservatives for the enzyme preparations, which were found to be stable for several weeks in the refrigerator.

DISCUSSION.

The action of the enzymes present in the culture supernatant fluids of certain strains of *Cl. welchii* (Type B) has been examined, using preparations of blood-group substances of animal and human origin as substrates.

The enzyme activities can be differentiated by heating the mixed enzyme preparations for 10 minutes at 55°, whereby the power to destroy the A and B serological character is lost. The capacity of the resulting solution to inactivate the H-substance is, however, unimpaired, and quite potent preparations of H-enzyme free from A- and B- activity can be obtained in this manner. Attempts to separate the enzymic activities by fractional precipitation techniques that involve the addition of ammonium sulphate, organic solvents or certain adsorbents to the crude culture filtrates have been, from a practical point of view, largely unsuccessful. Similarly, adsorption on charcoal columns failed to bring about a useful separation of the enzymes. By applying certain of these procedures, however, the specific activities related to total nitrogen or " tyrosine " were considerably increased. Nevertheless, no useful separation of the enzymes was attained, either from each other or from the depolymerase.

The properties of the enzymes most thoroughly studied are summarized in

Table V. It might be deduced from the results given there that the depolymerase could be freed from blood-group enzyme activity by dialysis, and that the depolymerase could be removed from the blood-group enzymes by allowing the preparations to stand at pH 11 for an hour at room temperature, but we have made no attempt to accomplish these changes.

TABLE V.—*Summarizing the Properties of the Blood Group Enzymes and Hog Mucin Depolymerase.*

Property.	A-enzyme.	H-enzyme.	Depolymerase.
pH optimum	5·5	6·5	6·8
Half activity, pH	4·9, 8·2	4·9, 8·3	5·6, 9·1
pH stability range	5–11	5–11	5·6–7·6
Temperature optimum	40–45°	40–45°	38°
Inactivation temperature (10 min.)	55°	60°	56°
Half activity, temperatures	25° 53°	25° 53°	33° 50°
Growth optimum (days)	1	1	1
Stability on dialysis	±	±	+
Stability on storage (liquid)	−	±	±
Average purification ratio (acetone)	200	200	200

The general lack of distinction between the physical properties of the blood-group enzymes is of interest. It could be surmised that certain groupings on the enzymic complex which are specific for the A- or B-substrate or responsible for the depolymerizing activity become denatured, leaving other groups unaffected and still able to bring about the inactivation of the H serological character. The evidence in support of such a conclusion is, however, far from complete. The inactivation by the enzyme preparation of the specific mucoids associated with the " Lewis " blood-group characters, Le^a and Le^b (Mourant, 1946 ; Andresen, 1947), has already been recorded (Grubb and Morgan, 1949).

The purified A-enzyme destroys the specificity of Group A mucoid when this character is measured by the haemolytic inhibition test, and in this property it differs from that shown by some crude enzyme preparations obtained from other types of *Cl. welchii* (Morgan, 1946) which failed to accomplish the destruction of A-specificity when measured by this technique. It is to be noted, however, that the differentiation of the two serological properties, the power to (*a*) inhibit iso-agglutination and (*b*) prevent the haemolysis of sheep-cells by rabbit serum, can be brought about by other means. Thus, it has been shown that after treatment with dilute acid the former activity is destroyed, whereas the latter activity is enhanced (Aminoff, Morgan and Watkins, 1948).

A strictly quantitative interpretation of the enzymic inactivation of the group substances in terms of the inhibition of agglutination is not straightforward when so little substrate remains, and when the indicator systems are relatively insensitive to changes in concentration of the reactants. The formula used to determine the units of activity when smaller or greater amounts than 94 per cent of the substrates are decomposed is based on the assumption that the reaction is not seriously affected by the almost complete destruction of substrate, and although primarily empirical, is of considerable practical value, and enables one to avoid making several titrations to determine the amount of the enzyme preparations required to bring about a lowering of the inhibition end-point by exactly four tubes.

Information about the chemical changes induced in the group active mucoids by the enzymes cannot readily be obtained when excessive amounts of crude enzyme material must be employed to destroy the serological character of these substances. The purified enzymes now described contain one-tenth as much nitrogen per unit as 1·0 mg. of the specific blood-group mucoid. They are, therefore, suitable for investigations of this kind and are being employed for this purpose. Similarly the action of the enzymes on the group specific character of stroma and intact erythrocytes is being studied.

SUMMARY.

The production from cultures of *Cl. welchii* of enzymes which destroy the specific serological characters of the human blood-group substances is described.

A quantitative method for measuring the activity of the enzymes has been applied to a study of their properties. The optimum pH values for the A- and H-enzyme activities are 5·5 and 6·5 respectively, and both enzymes are stable over the pH range 5–11.

A considerable purification of the enzymes was accomplished by the procedures described, and preparations containing 60 per cent of the original activity associated with 0·2 per cent of the crude filtrate nitrogen were obtained. These enzyme preparations, however, are not always stable when stored in the liquid or freeze-dried condition.

The serum of rabbits immunized with the purified enzymes developed weak anti-enzyme properties.

An additional enzyme which accompanied the group enzymes depolymerized an unidentified substrate in the "standard" preparation of hog gastric mucin. The enzyme is stable between pH 5·6 and 7·6, and shows an optimum activity at pH 6·8.

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THE SEROLOGICAL ANALYSIS OF MIXED FLOCCULATING SYSTEMS BY MEANS OF DIFFUSION GRADIENTS.*

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SEROLOGICAL analysis differs from other analytical procedures in that by itself it can demonstrate no more than a lack of homogeneity. The method to be described here combines specific flocculation with diffusion of antigen and antibody through a matrix so as to reveal the presence of individual systems within a mixture. The resulting flocculation can be identified by interactions with known flocculating systems.

The Limitations of Serological Characterization.

Detection of impurities.

The specificity of serological reactions needs no emphasis. Substances, such as the egg albumins of birds so closely related as the hen, turkey and guinea-fowl, which are beyond differentiation even by their electrophoretic mobilities, may yet be distinguished serologically. The weakness of serology is that in practice it is based on trial and error : a substance pure by chemical criteria is used to make an antiserum, which is then used to detect the homologous substance in solution. Nevertheless, because of serological sensitivity and specificity, "impurities" can in this way often be detected in preparations which have passed all other tests. There must be some preliminary idea, however, of the substances to be sought; for though the character of impurities may often be suspected from the nature of the original material and the methods of purification used, frequently the final and purest material obtained by physico-chemical means is a mixture which offers no hint whatever of its constituents.

Cross reactions.

There is another serological test for homogeneity. If one antigen reacts with serum prepared against a second antigen, both are regarded either as being identical or as containing an identical component, in the latter case the antigen being a mixture. If the first antigen is identical with the second it will, suitably mixed, remove all the antibody from the serum. For obvious reasons the reversed experiment will hold ; and a serum prepared against the first antigen should also behave similarly. The weakness of this approach lies in the fact that if both antigens are identical mixtures they may behave as though they were homogeneous.

* The subject matter of this paper formed part of a thesis submitted to the University of London in July, 1948, for the Ph.D. degree.

Irregularities in quantitative tests.

Multiple zones of flocculation by the tube method are accepted as evidence of a mixed system, but usually little can be learnt about the number of antigens present in the mixture, since closely spaced individual zones coalesce into a single broader zone. Furthermore, many of the observations on flocculative behaviour have been made with systems now known to be impure, and deductions so based may well be erroneous. It is doubtful whether a substance pure in the serological sense has so far been employed in the study of flocculative behaviour, for evidence of purity has always been chemical or serological and subject to the limitations outlined above. The purest substance, from the serological standpoint, which has been used for flocculation experiments is diphtheria toxoid. This is well known to show a rather odd flocculative behaviour and is regarded as an exception; it may well be that its behaviour is the rule and not an exception, the "rule" having been deduced from observations on mixtures.

Errors Inherent in the Tube Technique of Precipitation.

It is customary to carry out flocculation reactions with the volumes constant. Variation of the relative proportions of antigen and antibody is achieved by using a unit volume of one reagent and adding varying dilutions of the other. The fundamental observation concerning flocculation by the tube technique was that the velocity of the reaction was greatest at fixed proportions of the two reagents. That this indeed was of fundamental importance was further borne out by the fact that the optimal proportions by flocculation showed close agreement with the neutralization of toxins and with the complete removal of antigens showing no indicator effect. Closer investigation of various systems led to the unexpected fact that in some cases constant antibody (alpha) and constant antigen (beta) titrations showed different optimal proportions. This has been regarded as of theoretical importance, but as most of the observations were carried out in tubes with fixed volumes, the essentially artificial nature of the approach was not appreciated.

The limitations of the method become clearer if a hypothetical beta titration is examined (Fig. 1). In this it is assumed that one unit of antigen is equivalent to one unit of antibody, and therefore this combination has the greatest flocculation velocity. The steps of the antibody dilution are successively 1·5 times greater. Instead of the dilution factors, however, arbitrary antigen and antibody units have been used. It is clear that the 7th tube contains the two reagents in equivalent quantities. This is the tube therefore which will have the greatest reaction velocity (since the equivalence was chosen on this basis). Tubes on either side will show a lesser degree of precipitation representing reduced reaction velocity, until on either side a point is reached where the velocity is—for practical purposes—*nil*. Thus Tubes 1, 2 and 3 show no precipitation, and this is known as the zone of antibody excess. Similarly Tubes 13 and 14 are clear. This is the zone of antigen excess. The zone of precipitation is seen to be asymmetrical. If Tube 5 is now examined, a moderate degree of flocculation is found. At the time of mixing it contained 100 antigen and 200 antibody units, a ratio of 1 : 2. Assuming each unit of antigen to unite with one unit of antibody—a fact which seems to be warranted from the behaviour of the optimum Tube 7—by the time 99 units of antigen have united with 99 units of antibody the ratio would have

deteriorated to 1 : 101. A glance at Tube 3 shows that a ratio of 1 : 4 no longer flocculates, and consequently the reaction would have stopped—or taken a different course—long before the antigen excess of 1 : 101 was reached. Similar considerations apply to the antigen excess part of the precipitation zone. In Tube 11 the initial ratio of 4 parts of antigen to 1 part of antibody is compatible with flocculation, and may be thought of as gradually altering until theoretically the ratio of 76 units of antigen to 1 unit of antibody is reached. Tube 13 shows, however, that 8 units of antigen and 1 unit of antibody will no longer flocculate, and the reaction must stop long before attaining a ratio of 76 : 1.

In fact the composition of the precipitate varies with the proportions of the reagents, the reaction taking a different course in each tube. Each of these different reactions must have a reaction velocity smaller than that of the combination in the optimum tube, since the equivalence was arbitrarily chosen at

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Antigen	100	100	100	100	100	100	100	100	100	100	100	100	100	100	A
Antibody	800	600	400	300	200	150	100	75	50	37·5	25	18·75	12·5	6·25	G

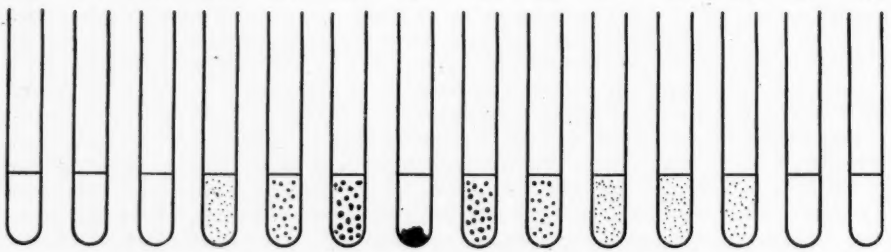


FIG. 1.—Constant antigen titration of a hypothetical flocculating system. The figures refer to units per unit volume, and it is assumed that one unit of antigen is equivalent to one unit of antibody.

the greatest velocity. It might be argued that the solubility equilibria of the compounds formed in the various tubes determine the result, but this view is not essentially different from the dynamic concept, and has the disadvantage of expressing results in terms that are not directly obtained from the experiment. This is the point of weakness of the tube method : that in a series of tubes an essentially different reaction takes place in each. In every tube, except the optimum, the system is under a distortion, which tends to become more accentuated as the reaction progresses. The true optimal ratio is not likely to be included in any set of titrations using a finite number of tubes, but will usually lie either to the right or to the left of the " optimal " tube, and the distorting effect described will come into play. These considerations already constitute a criticism of the tube method. If it is now assumed that the systems are multiple, involving several pairs of antigen and antibody, then there are several principal velocities, each surrounded by its own family of secondary velocities. The term " secondary velocities " is used to denote the reaction velocities occurring at other than optimal proportions. It is here suggested that the principal velocity

—the reaction velocity of the combination at optimal proportions of a given pair of reagents—is the fundamental feature of precipitin reactions. This may, however, be obscured in the case of multiple systems with optima so close to each other that a single zone is formed. The apparent optimum in such a case is misleading.

To sum up, the tube method is ill suited to the accurate determination of the velocity optimum of even a single theoretically pure system, and the characteristic velocity curves of multiple systems may be completely buried in what appears to be a single wide zone. However, it is intended to show how a system may be set up in which the particles of each reagent are free to choose their partners at any concentration, and the optimal proportions are obtained under dynamic conditions.

The Combination of Flocculation with Diffusion.

The law of optimal proportions manifesting itself in the form of a zone in the tube method formed an obstacle to the use of flocculation for the qualitative demonstration of either antigen or antibody. The earliest attempt to overcome the zone effect was the introduction of the ring test. When a serum is overlayered with a dilute antigen, the resulting precipitation is largely independent of the relative proportions of the reagents. The principle of this test is that an interface is formed between the antigen and antibody solutions, and from this plane antibody will diffuse upwards and antigen downwards. In this way concentration gradients will be set up, which, with the mixing involved in the process of overlayering, will produce conditions at some level including proportions capable of flocculation. This simple technique held the pride of place in precipitin reactions for the first quarter of this century, not because its principle was understood, but on account of its ready performance, and its applicability to the detection of minute amounts of antigen. Its circumvention of the zone phenomenon became obvious only in retrospect when the importance of the relative proportions in flocculation reactions had been recognized.

A gel as a matrix for combining diffusion with flocculation appears to have been first used by Bechhold in 1905. He incorporated gelatin into an antiserum, and after setting overlayered it with the antigen which was goat serum. After a time two sharply separated rings appeared which he believed to mean that the Liesegang phenomenon had been reproduced with colloids. Similar observations were reported by Reiner and Kopp (1927). The multiplicity of antigenic components which would afford an alternative explanation of such findings appears to have been completely neglected in the study of precipitin reactions until recently. Nicolle, Césari and Debains (1920) and Hanks (1935) mixed antigen with gelatin and overlayered it with serial dilutions of the antibody. Petrie (1932) combined nutrient medium with diffusion matrix. An antiserum was incorporated into nutrient agar on which colonies of homologous organisms showed surrounding rings of opacity, and streak inocula showed lines on either side. These opacities were thought to be due to flocculation between specific polysaccharide, liberated by autolysis, and antibody in the agar base itself. The nature of the reaction was confirmed by model experiments in which homologous purified polysaccharide was dropped on an agar base containing pneumococcal antiserum. Sia and Chung (1932) working independently on the *in vitro* transformation of types, used the same method. Petrie and Steabben (1943)

applied the method for the recognition of toxicogenic clostridia by using a medium specially suited for toxin production and adding antitoxin to it. Concentric rings were accepted as a positive reaction, and were believed to be due to the Liesegang phenomenon. Multiplicity of the toxins was not suggested as an explanation. They were less successful with *C. diphtheriae*, but Ouchterlony (1948) reported reasonably good agreement between this method and the guinea-pig test. In model experiments he showed that streak inocula across a ditch containing antitoxin yield lines which show joining. Elek (1948) obtained an antitoxin gradient by a filterstrip moistened with antitoxin and sunk into a medium specially designed for toxin production. It was found that this method

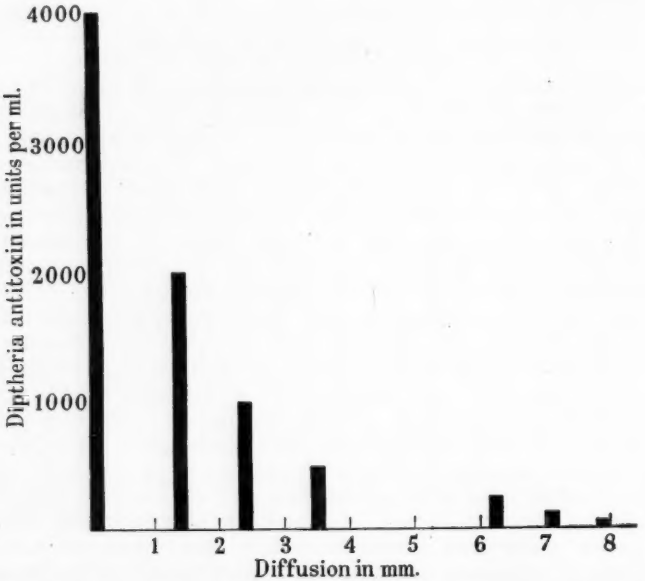


FIG. 2.—Diffusion gradient of diphtheria antitoxin in agar after 48 hours' incubation at 37° C.

was applicable to *C. diphtheriae* and to staphylococci. The latter gave multiple lines suggesting a multiplicity of antigens. This system was free from non-specific opacities which may occur on serum media. Carter and Wilson (1949) confirmed the practical usefulness of this test; Ouchterlony (1949) in a later paper also reported good results with a larger series which included ditch plates.

Thus diffusion can be utilized in two ways. One reagent can be incorporated into a matrix, and the other may be allowed to diffuse producing a concentration gradient. A system of this sort will be free from the zone effect as long as the concentration gradient includes the optimal proportions for the fixed concentration in the matrix. This, however, involves the preparation of several plates with various antitoxin concentrations, as in the methods of Petrie and Steabben (1943) and Ouchterlony (1948). The other way is to allow diffusion of both

reagents so that two concentration gradients are formed in the matrix. This may be referred to as the double-diffusion-gradient method, and has considerable advantages over the first in that it produces conditions not only for the interaction of antigen and antibody in all proportions, but also for the maintenance of a continuous and progressive reaction.

A concentration gradient of a diffusible reagent is produced by incorporating it at one point in the shallow layer of agar in a Petri dish. If a filterstrip soaked with a concentrated antibody or antigen is used, the concentration gradient due to diffusion can be visualized in the form of orbits, points of equal concentration being equidistant from the edge of the strip. In actual fact only a small part

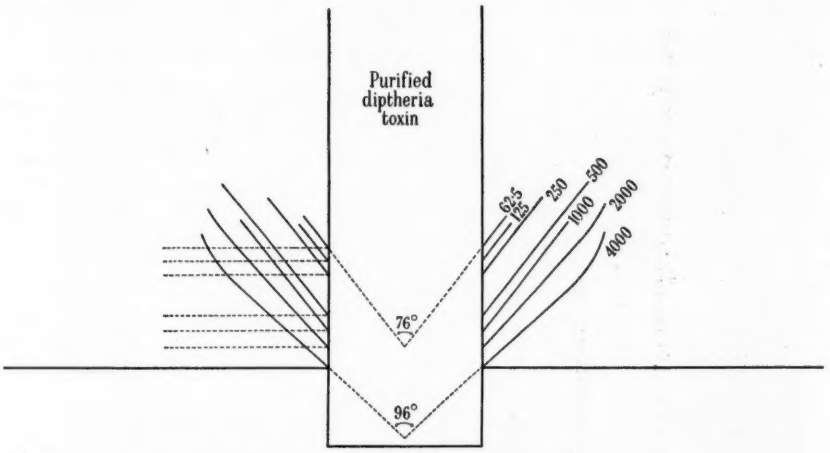


FIG. 4.—Composite diagram showing the effect of varying the ratio of antigen to antibody. The antitoxin was kept constant at 0·1 ml. of 4000 units per ml. The toxoid concentratino was varied from 4000 units per ml. to 62·5 units per ml., 0·1 ml. of each dilution being used.

of these orbits can exist on account of the shallow layer of the agar. The sections of the orbits will be at right angles to the surface, and for practical purposes lines parallel to the edge of the filterstrip represent equal concentrations. The only exception is the region next to the filterstrip. Here for a few millimetres conditions are different, as the position in depth of the strip influences the angle of the orbit and its face will not be at right angles to the surface. The effect of this tilting will be referred to later.

Fig. 2 shows the " observed " gradient for refined diphtheria antitoxin in a thin layer of agar. Diffusion is of course continuous, but the gradient shown illustrates the distribution of concentrations found after 48 hours' incubation at 37° C. Plates were poured with a standard amount of matrix, and a filterstrip measuring 10 mm. by 60 mm. containing exactly 0·1 ml. of refined commercial diphtheria antitoxin of a strength of 4000 units per ml. was placed into each plate.

After the setting of the agar a similar strip soaked with 0·1 ml. of purified toxoid of 4000 units per ml. was placed on the surface of the first plate at right angles to the other strip. Doubling dilutions of the toxoid were prepared, and a series of plates made by varying the concentration of the toxoid and keeping the antitoxin concentration steady at 4000 units per ml. Fig. 3*a* shows the result with 0·1 ml. of 500 units per ml. of toxoid, and Fig. 3*b* with 0·1 ml. of 4000 units per ml. It will be noted that the points of insertion of the lines are altered by different concentrations of the reagents. This method furnishes a convenient means of determining the concentration gradients in the plate. After 48 hours' incubation the plates were placed on photographic paper and contact prints were made. The lines thus obtained were superimposed and the diagram shown in Fig. 4 was obtained. The points of insertion of the lines and also the angles formed can be seen to vary. The variation is from 96° with 4000 units of toxoid per ml. to 76° with 62·5 units per ml. The point of insertion of any line into the toxoid strip represents the equivalent concentration of antitoxin at that point, assuming that over the area of the toxoid trip itself the concentration is uniform—a valid assumption for practical purposes. The distances of the points of insertion from the antitoxin strip can be measured and the antitoxin gradient thus plotted. Strictly speaking, as the lines are not formed quite simultaneously, the "observed" gradient will represent a slight distortion from the true gradient, but the actual difference in the values obtained is likely to be within the experimental error of the method. If lines parallel to the edge of the antitoxin strip be drawn from the points of insertion of the various flocculation lines in Fig. 4, these will represent antitoxin concentrations corresponding to the flocculation lines from which they originate. These lines will cut the other flocculation lines, and the points of crossing will yield the toxoid concentration at that point. In this way values

EXPLANATION OF PLATES.

FIG. 3*a*.—Line of flocculation produced by the double-diffusion-gradient method. Vertical strip contained 0·1 ml. of purified diphtheria toxoid, strength 500 units per ml. The horizontal strip contained 0·1 ml. of diphtheria antitoxin, strength 4000 units per ml. Incubation 48 hours at 37° C.

FIG. 3*b*.—Experiment as 3*a*, except that the vertical strip contained 0·1 ml. of toxoid, strength 4000 units per ml. Note that the line of flocculation is nearer to the horizontal filterstrip.

FIG. 9.—Flocculation of diphtheria toxin-antitoxin and toxoid-antitoxin systems demonstrating looping.

FIG. 10.—Plate showing various diffusible antigens by two different strains of *Staph. aureus*. Flocculation lines due to the same antigen show looping. One of the lines produced by "W. 46" can be seen cutting right across the line of growth of "CN 338," indicating that the latter does not produce this antigen.

FIG. 11*a*.—Plate showing lines produced by *Cl. welchii* type A with polyvalent anti-gas-gangrene serum.

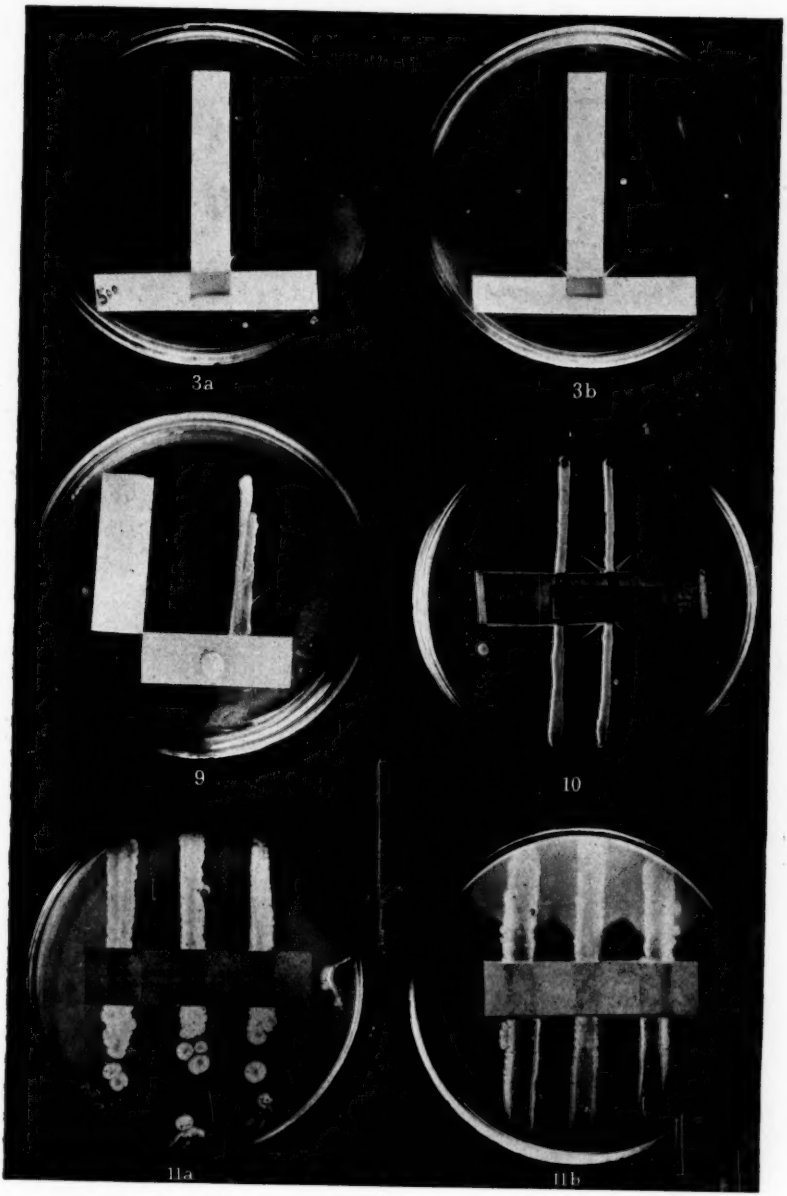
FIG. 11*b*.—Similar experiment to Fig. 11*a*, but incorporating human serum. The Nagler effect is clearly shown, and is demarcated by the flocculation lines furthest from the filterstrip.

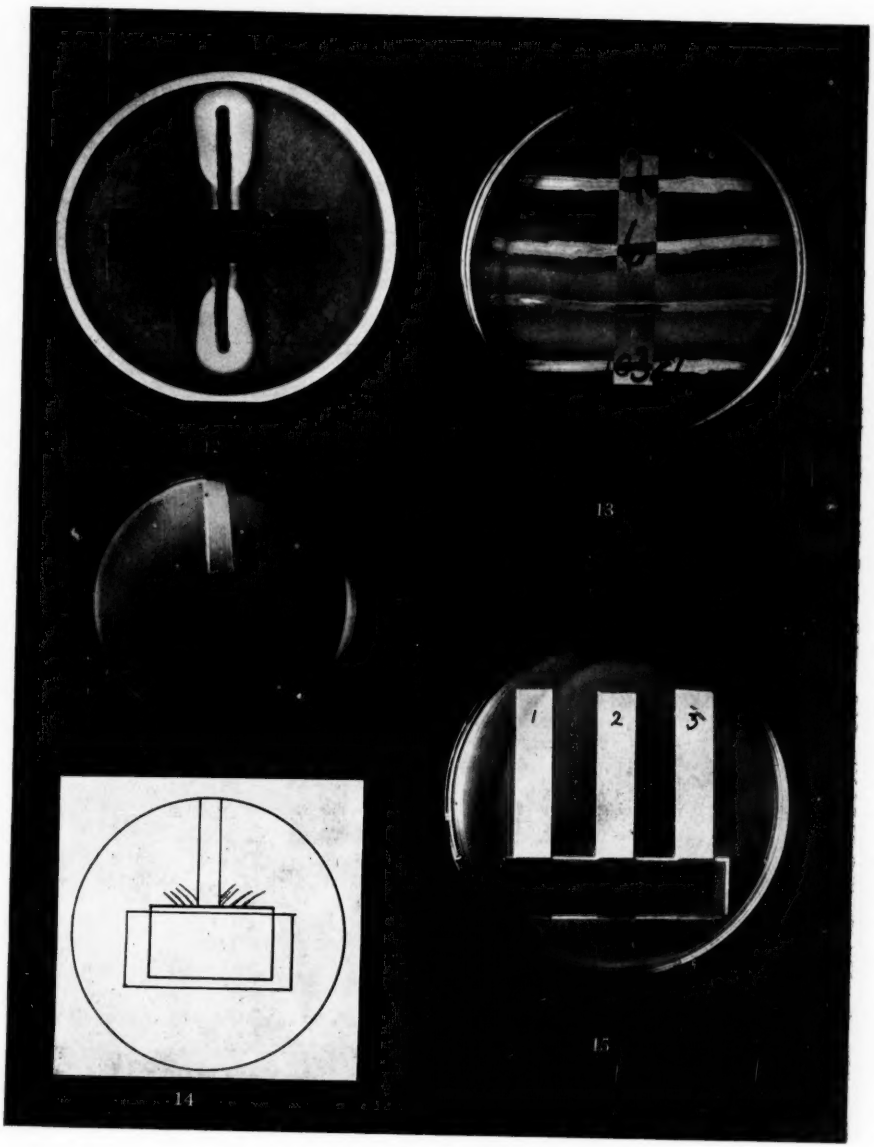
FIG. 12.—Agar plate incorporating sheep red cells showing a strain of *Staph. aureus* producing two haemolysins. One shows marked inhibition and the other very little.

FIG. 13.—Agar plate incorporating laked horse red cells inoculated with four strains of staphylococci. Strain "6" shows a turbidity which appears to be specifically inhibited by the antitoxin.

FIG. 14.—Antibody gradient produced by "feeding" the free edge of the filterstrip. The anti-human serum reveals five serological fractions in the human serum used as antigen.

FIG. 15.—Successive amounts of anti-human serum were dried into the horizontal filterstrips. The vertical filterstrips contained the following: No. 1 = "albumin" fraction of human serum; No. 2 = complete human serum; No. 3 = "globulin" fraction. This anti-human serum appears to be mainly anti-globulin.


3a
3b
9
10
11a
11b


13
1
2
3
14
15

for various toxoid and antitoxin concentrations are obtained and their distance from the strip edge can be measured. From the data thus obtained not only can the antitoxin gradient be plotted, but also the toxoid gradients at various initial values (Fig. 5).

This shows that both antigen and antibody can be incorporated in an agar base, so that a concentration gradient develops by diffusion for an appreciable distance. The range is theoretically from the original concentration used, at the strip edge, to *nil* at some distance from it. Although the actual rate of migration and consequently the shape of the gradiant may be different for

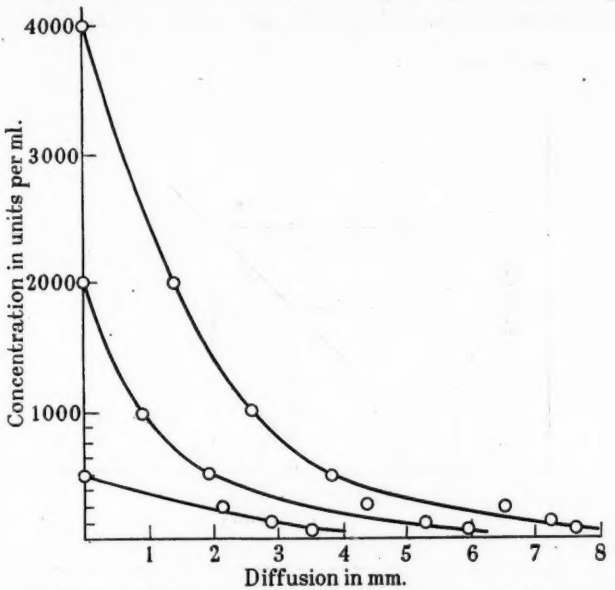


FIG. 5.—Diphtheria toxoid diffusion gradients in agar for different initial values after 48 hours' incubation at 37° C.

different antigens and antibodies, two gradients set at right angles to each other will lead to flocculation at optimal proportions along a plane which, viewed from above, appears as a line. The fact that what appears to be a line in the photograph is really a plane extending through the entire thickness of the medium can readily be seen in the actual plates if these are viewed by transmitted light, when the fine knife-edge of flocculation becomes quite obvious. The lines are seen to be broader at their insertion or may even appear split (see Fig. 3*a* and 3*b*), because the plane of flocculation is slightly tilted there as already described.

The conditions when two diffusion gradients are set at right angles to each other are shown in Fig. 6. For the sake of simplification the depth of the medium is disregarded and the diagram reduced to two dimensions. The first set of arrows issuing from the antigen and antibody strips, *a* and *a'*, represents their

concentrations at the end of a certain period of incubation. These concentrations represent equivalence, and at the point of the first pair of arrows precipitation occurs. After a further lapse of time equivalence again occurs at the point of the secondary pair of arrows, b and b', although the absolute quantity of the reagents is less. Under these conditions flocculation will occur along a continuous line. In the example chosen the antigen and antibody gradients are such that they have the same shape, and the initial concentration of the two reagents in the filterstrips is equivalent. These conditions produce a straight line passing at 45 degrees between the two strips and starting at the angle formed between them. In flocculation both antigen and antibody leave the system as they pass into the precipitated phase. Along the line therefore their respective concen-

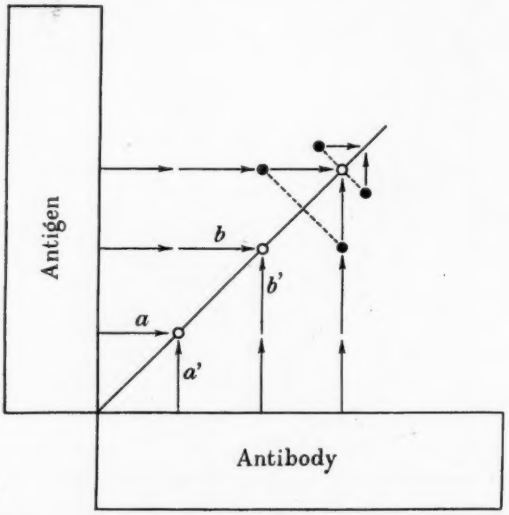


FIG. 6.—Diagrammatic representation of the dynamics of the double-diffusion-gradient.

trations are determined by the solubility of the precipitate, and this is for practical purposes *nil*. Particles equidistant from the line, shown in black, will migrate in the direction shown by the arrows, as the gradient has to be reconstituted between the zero concentration on the line and the concentration still existing in the filter-paper. However, the particles taking part in this new migration will also ultimately flocculate along the line. In this way a continuous flow of the reagents occurs towards the line which increases both in thickness and length. Thus the double diffusion gradient is not only free from the zone effect, but the dynamic conditions are such that the reaction proceeds continuously towards completion. When the initial concentrations of the reagents in the strips are not equivalent, or the gradients have a different shape, the line of flocculation will not pass through the angle of the strips or the line will not be straight. These conditions are a little more difficult to visualize, but the dynamics of the system remain essentially unchanged.

So far only theoretically pure flocculation systems have been considered, i.e. containing a single antigen and a single antibody. From the point of view of flocculation, however, a " pure " antigen and a mixed antiserum, containing amongst others the homologous antibody, will still represent a pure system. Likewise with a mixture of antigens and a single antibody directed against one of the antigens a pure system results in the sense that the combination of a single pair of reagents is believed to occur. These conditions are, however, seldom encountered in practice. Antigens used for the preparation of sera are biological products, and even though they may be purified for the flocculation reaction,

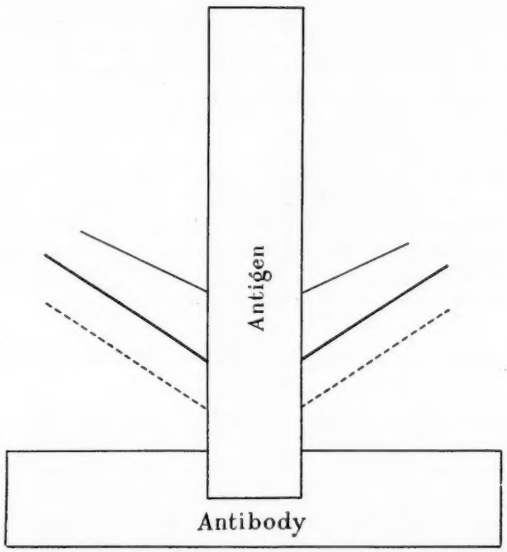


FIG. 7.—Resolution of mixed precipitating systems by the double-diffusion-gradient.

physico-chemical purification may not represent serological purity. Furthermore, even against a theoretically pure antigen several antibodies are possible. The utilization of the double diffusion method will usually lead to a separation of mixed systems. Fig. 7 shows the results with three hypothetical systems. The upper thin line, the middle thick line and the lower dotted line represent flocculation reactions. Each pair of reagents will produce its own diffusion gradients and will flocculate at its own optimal ratio. The conditions of the experiment militate against the overlap of the flocculation lines, as the position, shape and angle of each line will depend on the concentration and diffusion rate of the reagents involved. Although these three systems might show up as a broad zone by the tube method, the introduction of diffusion brings a new element which allows each to develop individually. Admittedly an overlap of the lines at their insertions may occur even under these conditions, but separation will occur further out when the differentiating effect of the diffusion comes into full play. To obtain complete overlap of two systems the same initial concentrations,

optimal proportions and diffusion velocities must be postulated for both antigens and both antibodies, and this is not likely to occur.

In these remarks concerning the separation of the lines it is assumed that the second stage of precipitation reactions is specific. The numerous examples of multiple flocculation systems, examined with the double diffusion gradient technique, confirm this assumption. Topley, Wilson and Duncan (1935) on the basis of agglutination experiments concluded in favour of Marrack's lattice hypothesis which postulates a specific second stage. They suggested that "the interesting implications of Marrack's hypothesis should be given due weight in future attempts to determine the mechanism of those antigen-antibody reactions in which flocculation plays a part." While with bacteria and red cells the question is comparatively easily approached experimentally, considerable difficulties arise with the identification of white precipitates of different nature. It will be seen that the double diffusion gradient enables one to identify a system with a known one, and thus furnishes striking proof of the specificity of the second stage in a field where proof was still required.

In most naturally occurring systems, such as toxin-antitoxin reactions, body fluid antigens, etc., we know that the antigen is a complex made up of several substances, the number of which may not be known. Likewise the serum may, and often does, contain multiple antibodies. The double diffusion gradient is comparable in its action to prism: the constituent systems are separated into individual lines, as are the colours of the spectrum.

The Application of the Double-Diffusion-Gradient Method to Toxin Analysis.

In recent years it has become increasingly evident that crude filtrates of organisms known to produce toxin may contain more than one poisonous antigenic factor. Thus the term "'toxin" shifted to denote these individual factors, and in some bacterial groups formed the basis of subclassification. The analysis of toxin pattern, although of great interest from the medical and veterinary point of view, is greatly handicapped by the technical difficulties encountered. Suitable indicator effects of the individual toxins have to be found, and large numbers of quantitative tests against various reference sera are required before the multiplicity of the factors can be established.

Determination of the number of lines.

Our knowledge of the toxic pattern is most complete for the clostridia, and especially *Cl. welchii* (Oakley, 1943). It seemed desirable therefore to observe the application of the method in this field. The experiment was carried out blindly, the constitution of the 8 sera used being unknown. The material was kindly provided by Dr. C. L. Oakley, of the Wellcome Physiological Research Laboratories, Beckenham, Kent. Streak inocula of the organisms were used for the toxin gradient, as in this way if satisfactory results were obtained they could more readily be applied to the laboratory identification of the types. The medium used had the following composition: Difco proteose-peptone 2 per cent, lactic acid (B.P.) 0·035 per cent, maltose 0·15 per cent, agar pulv. 2 per cent, pH = 7·6. This medium was tubed in 10 ml. quantities, and immediately before being poured was enriched with 0·5 ml. of horse serum and 0·5 ml. of Fildes' extract. Strips of

filter-paper, 60 mm. by 15 mm., soaked in undiluted clostridial antitoxin, were sunk into the middle of the plate before the medium solidified. Young broth cultures of the five organisms tested were centrifuged, and heavy streak inocula

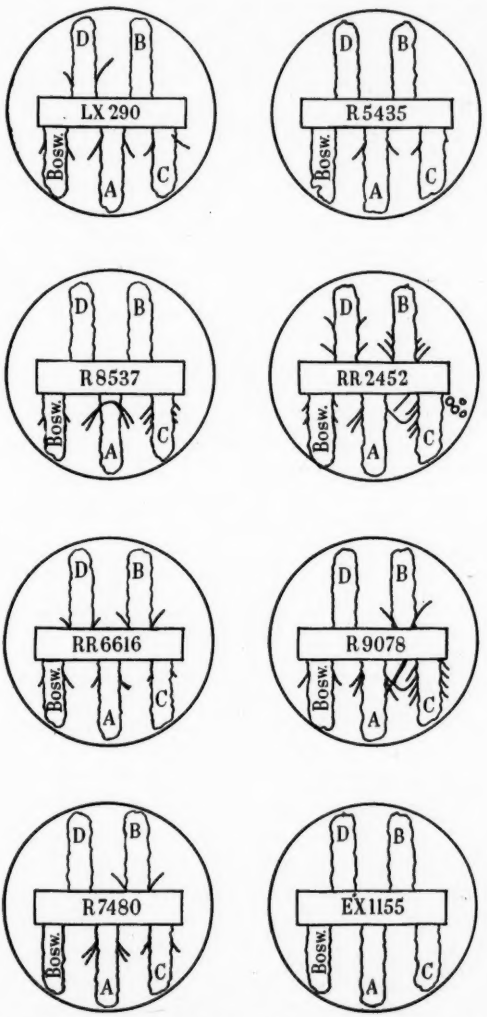


FIG. 8.—Flocculation lines produced by different types of *Cl. welchii* with various antisera.

made from the sediment in the manner shown in the diagram (Fig. 8). The plates were incubated in a McIntosh and Fildes jar for two days and final readings were taken after a lapse of several days at room temperature.

The data concerning the sera and toxic patterns were supplied after the conclusion of the experiment, and the information is shown in Tables I and II.

TABLE I.

Number.	Type of organism.	Toxins. α	β	γ	δ	ε	θ	ι	ϰ	λ
CN 1491	*Cl. welchii*, Type A, S. 107	++	−	−	−	−	+	−	+	−
CN 1990	,, Type B, S. 845	(+)	+	+	−	+	+	−	−	+
CN 1797	,, Type C, Strain Ashby	+	+	+	+	−	+	−	+	−
CN 1798	,, Type D, Strain R_2	+	−	−	−	+	+	−	−	+
CN 1241	,, Type Bosworth	+	−	−	−	−	+	+	+	+

TABLE II.

Serum.	Units of antitoxin. α	β	γ	δ	ε	θ	ι	ϰ	λ
LX 290	135	—	—	—	—	0·3	—	140	—
R 5435	0·2	—	—	—	—	190	—	55	—
R 8537	180	—	—	—	—	90	—	180	30
RR 2452	35	1250	—	4	180	45	—	?	?
R 6616	100	—	—	—	200	—	—	?	?
R 9078	75	3100	—	80	$<\frac{1}{5}$	700	—	?	?
R 7480	190	—	—	—	—	13	240	?	?
EX 1155	—	—	—	—	—	—	—	—	—

— Denotes lack of a measurable amount of antitoxin. ? Amount not determined.

Although the agreement with the expected findings is not absolute, the results are nevertheless striking. The two sera known to contain the highest number of individual antitoxins, R 9078 and RR 2452, give most lines with *Cl. welchii*, Type C, which in turn has the most complex toxin pattern. Serum EX 1155 was a normal human serum and yielded no lines at all. In attempting to assess the agreement, the limitations of the method should be stressed. The medium used may not yield all the toxins obtained in a cooked meat medium, and not all antigenic diffusible products are necessarily toxins. In spite of these limitations one practical application of the method is at once apparent; it seems possible to correlate the number of lines produced with the type of the organism. Thus with suitable modifications a method may be elaborated for the typing of *Cl. welchii*, based on the *number* of lines produced with various test sera.

The rule of looping.

It follows from the theoretical considerations already discussed that lines produced by identical systems must be continuous by this method, or in other words the lines thus produced must loop. Identical systems for these purposes are those in which either the antigen or the antibody or both are identical. Thus Fig. 9 shows looping between the toxoid-antitoxin line issuing from the vertical strip, and the toxin line issuing from a growth of virulent diphtheria bacilli. Although toxoid and toxin differ by their physiological effect showing a difference in chemical constitution, they unite with the same antitoxin and therefore behave like identical systems. Looping occurs because the points of optimum proportions for a single system must fall on a continuous line. As two lines approach each other a summation of the antigen concentration occurs in the region, and the

relatively larger amount of antigen will find its corresponding ratio of antibody nearer to the strip. This will lead to a bending of the lines towards the antitoxin strip and finally to their fusion, since for each antigen concentration there is only one optimal antibody concentration in the field. This only applies, however, if the lines are formed at the same time. Lack of looping—and consequently erroneous interpretation—may occur if one of the lines is already well formed when a second approaches it. The dynamic conditions of the double diffusion gradient are such that the concentration gradients suffer a deformity in the vicinity of a line after a while, and consequently the second line approaching it may show angular joining or none at all.

Looping is a practical means of identifying an unknown system with a known one. Furthermore, in the case of multiple lines produced by different strains looping will show whether the diffusible antigens are shared or not. Fig. 10 shows two strains of staphylococci tested against a serum known to be rich in antibodies to alpha and beta haemolysins. The medium used was Difco heart-infusion broth to which 0·2 per cent KH_2PO_4, 0·03 per cent $MgSO_4$ and 1·5 per cent agar were added, and the pH adjusted to 7·4. Plates were poured with the addition of 1 per cent horse red cells washed in saline, reconstituted and laked with saponin. The plate was incubated in 30 per cent CO_2 in air for two days, and photographed after four days at room temperature. Amongst others two heavy lines are issuing from strain " W.46." The antigen responsible for one of these is not produced by strain " CN 338 " and the line cuts across the streak of growth. Looping is shown by the other lines, revealing that these antigens are shared. Looping can thus be made use of in various ways. The diffusible antigen pattern, which includes the toxins, of organisms can be studied and compared. The individual systems can be further investigated by means of filterstrips soaked with known fractions of toxic filtrates.

The rule of overlap.

Some toxins have visible indicator effects which can be demonstrated in plates, e.g. various haemolysins, lipase, lecithinase and other turbidities. As the proportions for neutralization of a toxin and the optimal proportions for flocculation show close agreement, the indicator effect would be expected to stop abruptly short of the flocculation line. On one side of the line there is free toxin, capable of producing the indicator effect, and on the other side there is none. In fact it has been found that the edge of a visible indicator effect and the flocculation line coincide on the double-diffusion-gradient. This may be used for the identification of the toxin responsible for a line. Fig. 11*a* shows three strains of *Cl. welchii*, Type A, against commercial gas gangrene antitoxin on Hayward's (1943) medium, but without the addition of human serum. Fig. 11*b* shows the same experiment, but this time with human serum added. Two lines associated with each strain are visible on both of these plates, and they are probably due to the alpha and theta toxins. The boundary of the Nagler effect coincides with the lines further from the filterstrip, showing that these were the lines produced by the alpha toxin.

The method may find useful application when several haemolysins are produced by an organism, all acting on the red cells of the same species. Fig. 12 shows a sheep blood agar plate with a strain of staphylococcus. At least three different staphylococcal haemolysins are known to act on sheep red cells. The

strain shown produces a wide zone of haemolysis which is completely inhibited, but between it and the filterstrip another haemolysin shows which is only partially inhibited. As the serum used was known to have a high anti-alpha titre, it may be assumed that the line demarcating the wide-zoned haemolysis, which is further from the strip, is that of the alpha haemolysin.

The specific nature of a turbidity appearing on a plate may also be readily investigated by the double diffusion gradient method. Thus it was observed that some staphylococci grown aerobically on agar media containing laked horse red cells produce a marked turbidity around them. Fig. 13 shows four strains tested against a staphylococcal antitoxin. (The negative and positive signs refer to previous coagulase testing.) The first strain, 10381, shows no turbidity. The coagulase negative strain shows heavy turbidity around it, but it is not inhibited by the antitoxin. The coagulase positive strain shows no turbidity, showing that the effect is independent of coagulase. Strain " 6," however, shows a turbidity which is inhibited in the same wedge shape as were the Nagler effect and the alpha haemolysin shown in the previous pictures. Under the condition of the experiment at least two turbidity factors appear therefore, one which is inhibited by the antitoxin and one which is not. The former appears to be a hitherto undescribed indicator effect of one of the diffusible antigenic products of staphylococci.

The Application of the Double-Diffusion-Gradient to Serum Antigens.

Serum antigens generally yield broad zones with the tube method commonly employed, and also show the anomaly of different optima with the constant antibody (alpha) and the constant antigen (beta) titrations. As both these findings were considered to reflect the basic flocculative behaviour of such systems, it is of interest that multiple thin lines, similar to the toxin-antitoxin lines, can be obtained with serum antigens using two diffusion gradients set at right angles. The multiplicity of antigenic factors in sera is a fact well known from physico-chemical studies, but serological discrimination is likely to go yet further. Oudin (1947) incorporated rabbit serum prepared against horse serum in an agar base, and overlayered it with the antigen and various chemically separated fractions of it. Multiple planes of flocculation appeared, as in the case of Bechhold's experiments, and these were interpreted as representing serological fractions. In this type of experiment the two diffusion gradients are set in opposition to each other, i.e. at 180°, and the dynamic conditions resulting are unsatisfactory. As flocculation proceeds the ratio of antigen to antibody on either side of the plane of flocculation becomes altered. Finally a gross excess of one of the reagents will cause the floccules to dissolve, and the plane of flocculation will appear to move on. The planes thus produced are fuzzy and difficult to observe for prolonged periods. When the two diffusion gradients are set at 90°, however, the planes of flocculation are well defined and stationary as the dynamics of the system produce a continuous and progressive reaction.

The difficulty in setting up a right-angled double diffusion gradient, similar to the one used in toxin experiments, is in bringing enough antibody into the system. When a serum is used for immunizing, vastly different titres of antibody may appear against the different antigens contained in it. The titre usually determined refers merely to the major antigenic component. An antiserum with an apparently high titre therefore may be deficient in antibodies against the other

antigens, and to reveal their presence an increased concentration is required. Various means can be used to increase the amount of antibody in the gradient. One method is shown in Fig. 14, and may be described as a " feeder plate." It shows the behaviour of human serum against a rabbit anti-human serum. The plate was poured with 2 per cent agar with 0·5 per cent salt (not nutrient agar, to reduce contamination), and a wide piece of filter-paper was embedded *vertically* and held in position until the agar set. A microscope slide cut down in length was placed adjacent to it and the strip bent over it. Several drops of anti-human precipitating serum were placed on the strip, the glass slide underneath preventing the antibody from leaking on to the plate. Finally a narrow strip of filter-paper soaked in human serum was placed at right angles to the first strip on the surface of the agar. The plate was incubated for three days, further drops of antibody being placed daily on the free edge of the strip. This arrangement produces a good antibody gradient with a weak serum. The wide strip is acting as a wick, and the total amount, rather than the concentration, of antibody entering the gradient is increased. The diagram shows the development of two heavy, two fine, and one very fine line, suggesting that there are at least five different systems. The individual existence of these cannot be demonstrated by the tube method.

Another technique is to increase the antibody concentration in the strip by drying-in successive amounts. Fig. 15 shows the application of this method. Three filterstrips were repeatedly soaked with the antibody and dried *in vacuo*. In this way an increased volume of antiserum was incorporated in the strips and the initial concentration raised. The three strips superimposed were sunk into a salt-agar matrix. This arrangement obviates the objection of intermittent feeding with antibody and the possibility of producing waves. Human serum was separated into two fractions using Pillemer and Hutchinson's (1945) methanol technique. The vertical strip shown on the left of the picture was soaked in the " albumen " fraction, the middle in normal human serum, and the one on the right in the "globulin " fraction. The plate showed an almost identical pattern with normal serum and the globulin fraction, while the albumen fraction showed only a fine haze. This particular anti-human serum appears to be predominantly anti-globulin.

Methods utilizing the right-angled double diffusion gradient principle offer possibilities for the investigation not only of antigen mixtures, but also of different antibodies. Nor is the method limited to toxin or serum-antiserum systems. Recently it was found possible to demonstrate the presence of at least two impurities in a highly refined preparation of diphtheria toxoid and this finding was in good arrangement with the electrophoretic pattern, which also showed two added substances.

DISCUSSION AND SUMMARY.

In the foregoing an approach to the characterization of macromolecules capable of acting as antigens or haptens has been described. It is based on the combination of diffusion and specific serological flocculation. The principle is to place two diffusion gradients at right angles to each other. It is claimed that under these conditions flocculation will occur at optimum proportions under dynamic conditions, and the reaction will tend to move towards completion. These conditions will be free from some of the limitations and disadvantages of

the tube method commonly used in serology. Furthermore the arrangement described reveals the nature of complex flocculating systems by spatial separation of the individual antigen-antibody reactions, and thus yields a new method for the investigation of homogeneity.

The examples illustrating the use of the double-diffusion-gradient were drawn chiefly from the field of bacterial toxins, where its usefulness is most immediately obvious. With suitable adaptations, involving the design of the matrix for high toxin yields, the method may be used for the detection of toxin-producing bacterial strains in routine work. It is also capable of extension to the analysis of multiple toxins, and the toxin-pattern of strains can be investigated by this relatively simple method.

The double-diffusion-gradient is essentially a qualitative approach effecting only a separation of the individual systems. The agar technique described in Petri dishes is, however, only an application of the principle, in the same way as filter-paper chromatography is an application of chromatography itself. The different planes of flocculation should be capable of production on a larger scale than possible in the shallow layer of a Petri dish. These planes of precipitation could be physically separated and further analysed, as they are likely to represent a purer combination than the floccules obtained by the tube method.

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SPONTANEOUS MUTATION TO STREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI*.

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MANY theories have been advanced to explain the increase in resistance of bacteria which frequently follows their exposure to an antibiotic. The view that the action of the antibiotic is selective, killing all but resistant bacteria which have arisen by mutation from sensitive bacteria, is now widely accepted. The critical experiments of Luria and Delbrück (1943), which showed that virus resistant bacteria arose by mutation from sensitive bacteria independently of the action of the virus, supports this view. Studies of the type carried out by these authors give not only quantitative information about the incidence of variant bacterial strains, but also provide a means of studying the problem of mutation in living cells. In view of this it seemed worth while to apply the methods of Luria and Delbrück to another strain of the bacterium they studied, *Escherichia coli*. Consequently tests were carried out to determine—

(1) Whether *E. coli* highly resistant to streptomycin occurred in cultures which had not been exposed to streptomycin.

(2) Whether resistant colonies retained their resistance on subculture and were all of the same type.

(3) The mutation rate from sensitivity to full resistance.

Other aspects of this general problem are being investigated in this laboratory by Newcombe and Harwirko.

MATERIALS AND METHODS.

Materials.

Escherichia coli strain B/r (Witkin, 1946, 1947) was the organism studied, all test cultures being inoculated from a single bacterial suspension.

Two media, Difco nutrient broth with sodium chloride added to a concentration of 5 per cent and a synthetic medium (M_9 of Anderson, 1946) were used for test cultures.

For testing resistance, 10,000 μg. of streptomycin sulphate (Lilly) were spread on 10 ml. of agar in Petri dishes. These were stored overnight at 5° C. to permit diffusion. The final concentration of streptomycin averaged 1000 μg. per ml.

Experimental procedure.

The procedure was a modification of that used by Luria and Delbrück. Eight or ten cultures, started from inocula so small (100 to 1000 bacteria) that there was little chance of a resistant bacterium being included, were incubated for 48 hours.

With all M_9 cultures, air was bubbled through the tubes during the last 24 hours in order to increase the population. The incubation temperature for plates and cultures was in all cases 37° C.

The final numbers of bacteria were estimated from plate counts. In the case of the resistant bacteria a suspension of the centrifuged culture, complete except for the small sample (0·1 ml.) taken for the total assay, was plated on streptomycin agar. The large number of bacteria plated, formed a milky film, which remained unchanged during 48 to 72 hours' incubation except for the development of colonies on certain of the plates.

A representative number of the surviving colonies were suspended in sterile saline and tested for resistance in the following manner. Equal quantities of the suspension were plated on plain and streptomycin agar, incubated and colony counts made. The resistant colonies fell into two categories.

(1) *Resistant* independent (*r*), (colonies on both plates).
(2) Resistant *dependent* (*d*) (colonies on streptomycin containing agar only).
The terms *resistant* and *dependent* will be used for these.

Calculations.

All calculations are by the methods of Luria and Delbrück (1943). These include a test of the hypothesis that resistant bacteria arise by mutation and the calculation of the mutation rate (a) *the probability of occurrence of a mutation per bacterium per bacterial generation.* Two methods are available for the latter, one which uses the proportion of cultures with no resistant bacteria (p_o) and one which uses the average number of resistant bacteria per culture (r). Values of the total population (N_t) and the number of test cultures (C) are also needed. The three formulae are as follows, Formulae 1 and 2 being in the form given by Newcombe (1948).

(1) $a = -(\log_e 2)(\log_e p_o)/N_t$.
(2) $r = (a N_t/\log_e 2)\log_e (C a N_t/\log_e 2)$.
(3) $\text{Variance}_r = C a^2 N_t^2$.

EXPERIMENTAL.

Sixty cultures tested in the above manner are described in Table I. Twenty-four of them contained bacteria which produced colonies on streptomycin.

Twenty of the resistant colonies were then tested in the manner described. Of these, fourteen were *resistant* and five *dependent* (Table II). *Resistant* and *dependent* strains have now been observed in many normally sensitive species (Miller and Bohnhoff, 1947 ; Paine and Finland, 1947 ; Yegian and Budd, 1948 ; Spendlove, Cummings, Fackler and Michael, 1948 ; Hobby and Dougherty, 1948 ; Alexander and Leidy, 1947).

A variant from the parent strain which arises spontaneously and passes on its new characteristics to succeeding generations, is termed a mutant. Furthermore, as has been pointed out by Luria and Delbrück (1943), variants which are mutants are likely to be distributed throughout a series of small cultures with considerable culture to culture difference in frequency. The present series with one giving rise to 815 resistant colonies ; three to more than 20 ; the remainder to less than 10, displays this characteristic. Values of a function of this *variance*

TABLE I.—*The Frequency of Occurrence of Resistant Bacteria in Cultures of* Escherichia coli *Strain B/r (Witkin, 1936).*

Experiment.	A.	B.	C.	D.	E.	F.	G.
Number of cultures	10	10	8	8	8	8	8
Culture medium	Broth	Broth	M_9	M_9	M_9	M_9	M_9
Volume of culture (ml.)	10	10	10	10	10	10	10
Average number of bacteria in inoculum	?	8	161	210	126	86	78
Average end number of bacteria per culture, $\times 10^9$	2·4	1·9	32	22	29	20	26
Numbers of resistant bacteria in individual cultures:	1	0	0	0	0	0	0
	0	2	0	4	1	0	0
	0	0	0	4	1	0	0
	0	0	3	3	6	0	0
	1	4	0	2	2	2	0
	0	0	815	0	1	0	40
	0	27	9	7	0	0	2
	0	0	0	8	0	29	3
	0	0					
	0	0					
Data concerning mutation to resistance:							
p_0	·800	·700	·625	·250	·375	·750	·625
r	·2	4·1	103	3·5	1·4	3·9	5·6
Rate (Formula 1) $\times 10^{-11}$	6·4	13·0	1·0	4·4	2·4	1·0	1·3
Rate (Formula 2) $\times 10^{-11}$	6·8	45·7	42·2	4·4	1·8	5·3	5·3
Stand dev./r exper.	2·00	1·96	2·61	0·78	1·34	2·44	2·33
Stand dev./r calc.	2·58	0·67	0·37	0·78	1·06	0·77	0·70

in the number of mutants per culture (the *standard deviation* divided by the *mean*) are given in Table I. These may be compared with the value of the same function to be expected if the resistant cells arise by mutation (Formula 3). The present data confirm those of Luria and Delbrück (1943) in that the experimental value is even greater than that predicted. It is therefore suggested that the resistant colonies are descended from resistant cells which have arisen by mutation in the test cultures. Alexander and Leidy (1947) take a similar view of their streptomycin resistant strains of *H. influenzae* Type b.

The estimated values of the mutation rate (Table I) range from 1×10^{-11} to 13×10^{-11} by Formula 1, with an average of $4{\cdot}4 \times 10^{-11}$; and from $1{\cdot}8 \times 10^{-11}$ to $45{\cdot}7 \times 10^{-11}$ by Formula 2 with an average of 16×10^{-11}. The value by Formula 1 is generally the lower. A difference of similar magnitude and in the same direction was also observed by Luria and Delbrück (1943), Demerec and Fano (1945), and Newcombe (1948), and this has been shown by the latter to be due to a downward bias with Formula 1. It is therefore likely that the higher

TABLE II.—*Assays of Streptomycin Survivors.*

Colony tested.	Plate counts.		Classification of tested colony.*
	Agar.	Agar and strept.	
1	91	121	r
2	360	320	r
3	17	20	r
4	0	12	d
5	2139	2429	r
6	169	190	r
7	83	74	r
8	2549	3100	r
9	1345	1374	r
10	15	19	r
11	0	99	d
12	0	45	d
13	157	151	r
14	0	492	d
15	142	0	s
16	0	184	d
17	35	59	r
18	184	142	r
19	9	13	r
20	492	263	r

r = resistant; d = dependent; s = susceptible.

value by Formula 2 is nearer the truth. But the latter formula is strictly applicable only when growth rates of parent and mutant strains are the same, a condition which is unfulfilled whenever *dependent* bacteria are present for, according to Newcombe and Hawirko (1949), such bacteria undergo at the most only two divisions in streptomycin free broth. Consequently values by this formula also suffer from a downward bias and it is likely that the mutation rate is higher than 16×10^{-11} and that a resistant bacterium will arise at the division of a sensitive one when the population approaches ten thousand million. This is of the same order as the mutation rate to streptomycin resistance in *H. influenzae* Type b (4×10^{-11}) (Alexander and Leidy, 1947), and lower than that to phage resistance in *E. coli* (1×10^{-8} or higher) (Demerec and Fano, 1945).

DISCUSSION.

The foregoing data are consistent with the view that resistance to streptomycin in *E. coli* strain B/r arises as the result of the mutation of sensitive bacteria. Consequently resistant bacteria will appear with a characteristic frequency (the mutation rate) in susceptible populations independently of the presence of streptomycin. Once such a mutation has taken place, the bacterial population will contain at least one bacterium capable of growth in the presence of streptomycin. Addition of streptomycin will destroy the susceptible bacteria, leaving the resistant ones and this will not be remedied by further administration of streptomycin. Withdrawal of streptomycin on the other hand may be destructive to the resistant

population if the bacteria are of the dependent type. To destroy independent ones an antibiotic other than streptomycin must be sought.

Reports of research in which streptomycin has been given in combination with another substance are of special interest in this connection. Smith, McClosky, Jackson and Bauer (1947) report greater recovery from tuberculosis in guinea-pigs when treated with streptomycin and a sulphone compound than when treated by either substance alone. Encouraging results were obtained by Lincoln (1948) following treatment of patients suffering from tuberculous meningitis and miliary tuberculosis with streptomycin and promizole. A study of the effect of other antibiotics on the resistant strains should be of value in discovering additional combinations of therapeutic value.

SUMMARY.

(1) *Escherichia coli* strain B/r resistant to 1000 micrograms per ml. of streptomycin can be isolated from populations of the order of 1×10^{10} bacteria by plating in the presence of the drug.

(2) These are of two types, one of them requiring streptomycin for growth, the other not.

(3) The highly variable distribution of these resistant bacteria throughout the series of cultures examined is consistent with the view that they arise by mutation.

(4) The mutation rate for the change from susceptibility to resistance is of the order of 1×10^{-10} per bacterium per bacterial generation.

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BLOOD HISTAMINE IN THE ANAPHYLACTOID SHOCK PRODUCED BY ANTI-PLATELET SERUM IN DOGS.*

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The work of Dale and Laidlaw (1919) and Dragstedt and Gebauer-Fuelnegg (1932) showed histamine to be the aetiological agent responsible for anaphylactic shock, this substance being found in sufficient concentration in the blood to explain the majority of the observed signs. High levels of blood histamine were also found in peptonic (Dragstedt and Mead, 1937) and tryptic (Arellano, Lawton and Dragstedt, 1940) shock.

It was recently shown by one of us (Cruz and Silva, 1949) that the classical symptoms of the anaphylactic reaction could be produced in guinea-pigs, rabbits, rats and dogs by the intravenous injection of the appropriate anti-platelet serum. This phenomenon has been submitted to more detailed analysis, and this paper presents the results of experiments designed to study the variations of the blood histamine concentration during anti-platelet shock in dogs.

MATERIAL AND METHODS.

Dogs, previously anaesthetized by thionembutal, were used for these experiments. The carotid blood pressure was recorded by a mercury manometer and samples of blood for histamine estimation were taken either from the jugular vein or femoral artery three times in each experiment—before the injection of the anti-platelet serum, after the injection of the serum when the blood pressure fell, and 8 to 15 minutes after the blood pressure reached its lowest level. It was sometimes impossible to take this last sample owing to the low level of the blood pressure or the death of the animal. The concentration of histamine in the blood was determined by the method of Code (1937). The amount of anti-platelet serum injected varied with its titre according to Tocantins' method of dosage (1936); the dose usually varying between 2 and 4 c.c. per kg. body-weight, corresponding to titres of serum 1 : 16 and 1 : 32. The histamine concentration was expressed in terms of histamine base.

RESULTS.

The blood concentrations found are shown in Table I.

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TABLE I.—*The Histamine Content of the Blood of the Dogs before and after the Injection of Anti-platelet Serum.*

Number of dog.	Histamine before injection of anti-platelet serum (μg. per c.c.).	Histamine 2 min. after injection of anti-platelet serum (μg. per c.c.).	Histamine 8–15 min. after injection of anti-platelet serum (μg. per c.c.).
1	0·133	0·120	—
2	0·150	0·133	0·086
3	0·160	0·185	0·172
4	0·030	0·030	0·036
5	0·207	0·256	—
6	0·240	0·300	—
7	0·073	0·070	—
8	0·150	0·135	0·042

The figures in Table I show that there was no significant difference between the blood histamine level before and after the injection of anti-platelet sera. In no experiment was a discharge of histamine observed such as is encountered in anaphylactic and peptonic shock. The injection of the antisera even caused death in some of the animals without there being any significant variation in the blood histamine concentration. These results show that anti-platelet shock in the dog is not due to the liberation of histamine.

CONCLUSIONS.

The anaphylactoid shock produced in the dog by the injection of anti-platelet serum was not accompanied by any change in the concentration of histamine in the blood.

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